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VOLUME 3 JANUARY, 1957 No. 1

THE JOURNAL OF GENERAL AND APPLIED MICROBIOLOGY

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Published Quarterly By

THE INSTITUTE OF APPLIED MICROBIOLOGY

THE UNIVERSITY OF TOKYO

TOKYO JAPAN

COMMONWEALTH MYCOLOGICAL INSTITUTE
7 OCT 1957

The Journal of General and Applied Microblology is issued quarterly by the Institute of Applied Microbiology, University of Tokyo.

The journal is distributed without charge to, preferably in exchange with similar publications from, outstanding scientists and scientific organizations interested in microbiology and related fields.

Correspondence concerning the journal should be addressed to the Institute of Applied Microbiology, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

J. Gen. Appl. Microbiol.
Vol. 3, No. 1, 1957

A HIGH FREQUENCY OF HETEROZYGOUS DIPLOIDS AND SOMATIC RECOMBINATION INDUCED IN IMPERFECTI FUNGI BY ULTRA-VIOLET LIGHT*

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Received for publication Dec. 1, 1956

It was discovered for the first time by Pontecorvo and his co-workers[1-7] that fungi such as *Aspergillus niger* and *Penicillium chrysogenum* have a so-called parasexual life cycle instead of an orthodox sexual one. Ishitani, Ikeda and Sakaguchi[8-9] have found that the case is almost the same in *Asp. oryzae* and *Asp. sojae*, which play an important part in Japanese fermentation and food industries.

This paper is concerned with an attempt at increasing the frequency of diploidization to a degree sufficient to analyse the genetic background of the fungi and to improve industrial strains. We have succeeded in increasing the frequency to the order of a few percent of the survivors by irradiating heterocaryons with ultraviolet light; diploids of spontaneous origin in these fungi are believed to be found in a frequency of about one in a million.

EXPERIMENTAL

1. *Strains*

The strain used in this investigation is *Asp. oryzae* 51. Two of its mutants, one a white leucineless and the other a yellow lysineless, were produced in sequence by ultraviolet irradiation. Heterocaryons between these two mutants were recovered as green prototrophic conidia, containing an average of 5.7 nuclei. These heterocaryons segregate both component types in a frequency of 80 percent when they produce conidia again.

2. *Media*

The following media, with or without agar, were employed for the experiment.

Minimal medium; glucose 3%, $NaNO_3$ 0.2%, K_2HPO_4 0.1%, KCl 0.05%, $MgSO_4 7H_2O$ 0.05%, $FeSO_4$ 0.001%.

Complete medium; Malt extract containing about 8% of sugars (Balling 8°) was employed as a complete medium.

* This paper was read at the meeting of 10th International Genetics Congress at Tokyo, on Sept. 7, 1956.

Supplemented media; Three types of modified minimal media, supplemented with 100 mg per 1000 ml of L-leucine or L-lysine or both of L-leucine and L-lysine, were prepared to identify the growth factor requirement of the mutants and their recombinants.

Limited medium; A medium containing Koji extract (Balling 2°) and agar (3%) was employed for heterocaryon formation. The conidial mass arising from two different growth factor-requiring strains were cross-streaked densely on the agar plate and the resulting conidia were harvested after 7–10 days to isolate prototrophic heterocaryons with minimal medium.

3. *Diagnosis of a diploid*

Under the assumption that the original strain and its mutants are haploids, a strain being derived from the combination of the haploids and being

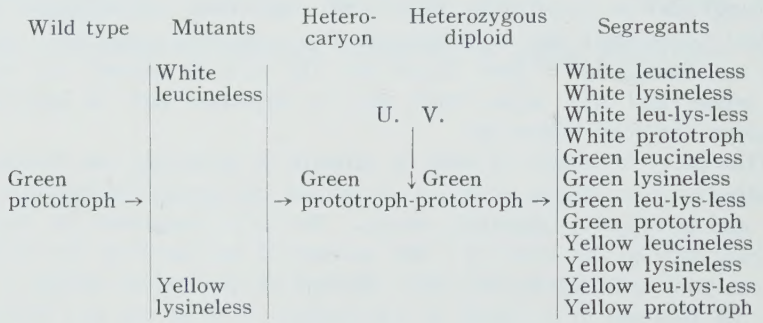
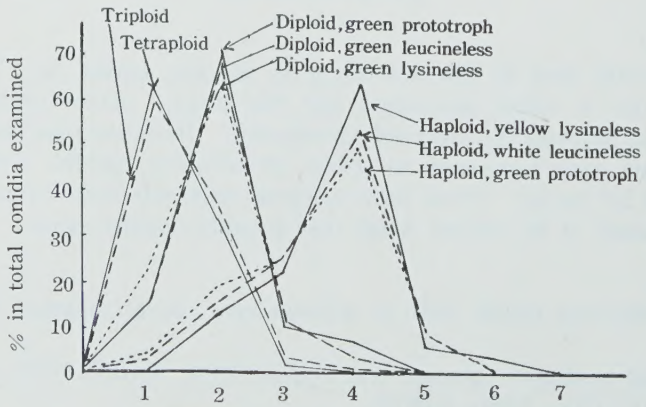


Fig. 1. Heterozygous Diploids and Somatic Recombination in *Asp. oryzae*



The staining technique was described in detail in the preceding papers[10].

Fig. 2. Distribution of Nuclei in Haploid, Diploid, Triploid, and Tetraploid Conidia

responsible for the following conditions was considered to be a diploid.

(1) In diploids, most of the conidia remain green and prototrophic even after sequential transfer; in contrast, the heterocaryon segregates its components at a comparatively high frequency.

(2) If segregation occurs in a diploid, some of the segregants should be recombinants as already demonstrated by Pontecorvo and his colleagues.

(3) In *Asp. nidulans*, the size of conidia increases with ploidy, but in *Asp. oryzae* and *Asp. sojae* this is not the case. The size of conidia is the same in haploids and diploids, but the number of Feulgen stainable nuclei[10] decreases from an average of 3.5 in the haploid to 1.9 in the diploid, indicating that in multinucleate fungi such as *Asp. oryzae* and *Asp. sojae* the absence of increase in conidial size is compensated for by a decrease in nuclear number. About this strange phenomenon, we have already published our opinion with data on the DNA content per nucleus[11].

4. *Check for the spontaneous diploidization and the back mutation*

Taking these diploids features into account, the frequency of spontaneous diploidization was estimated by plating heterocaryotic conidia in minimal medium, but no diploid colony was discovered among 5×10^4 colonies that were prototrophic and might have been either heterocaryotic or heterozygous diploid. Accordingly, the frequency of naturally occurring diploids may be 10^{-5} or less.

In addition to this, we have checked the frequency of back mutation by

Table 1. Spontaneous Diploidization and Back Mutation

1) Spontaneous diploidization

Exp. No.	Colonies observed	Diploid colonies	Diploid / Total
1	44.000	0	—
2	4.007	0	—
3	268	0	—
Total	48.275	0	0/48.275

2) Back mutation (after irradiation, 2 min.)

Strains	Markers tested	Frequency of back mutation	
		Spontaneous	Induced
White leuciness	*w*→*W* *leu*→*LEU*	0/1.715 0/16.200	0/9.321 0/23.900
Yellow lysineless	*y*→*Y* *lys*→*LYS*	0/1.386 0/14.000	0/13.586 0/35.000

plating the parental mutant strains on complete medium after ultraviolet irradiation. No back mutation was found in our tests.

5. *Enhancement of heterozygous diploid formation by ultraviolet irradiation*

Now let us see the effect of ultraviolet light on diploidization. In order to prepare a cell suspension containing about 100,000 conidia per milliliter, a conidial mass harvested from a heterocaryon was vibrated vigorously in sterile water and filtered through paper. With the assurance that more than 90 percent of the conidia were single, they were exposed to ultraviolet light from a 15 watt germicidal lamp at a distance of 33 cm. It is not so hard, after a little training, to tell, with the naked eye, a diploid colony from a heterocaryotic one, since in a diploid colony the whole surface is covered with green conidia, whereas in a heterocaryotic colony three types of sector, white, yellow and green corresponding to the components and the heterocaryon, can be easily detected. As shown in Figure 3, the frequency of

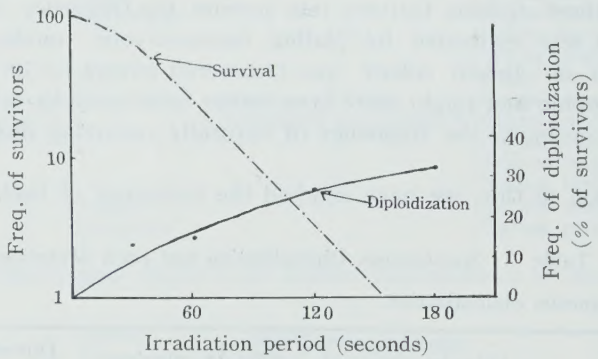


Fig. 3. Enhancement of Diploidization of Heterocaryotic Conidia with Ultraviolet Light

diploidization increased with the dose, reaching 32.6 percent of the survivors and 0.3 percent of the total heterocaryons irradiated. These values were reproduced several times in *Asp. oryzae* and *Asp. sojae*, but whether or not ultraviolet light has the same effect upon other fungi still remains untested.

6. *Evidence of somatic recombination*

Notwithstanding the low frequency of segregation, a diploid colony developed white or yellow sectors after a long cultivation. Then, the whole colony of a diploid strain was harvested after 7 days and served for the isolation of segregants.

Tow methods were employed for the purpose. The left in Figure 4 is a direct selection method, suitable for the isolation of colored segregants. The right is a filtration method, suitable for the enrichment of growth facor-

requiring segregants. Colored colonies were picked up intentionally, but requirers were picked up at random because we did not know what the requirement was. The requirement was determined with the presence or absence of growth on the media illustrated in the figure.

Table 2 shows the number of segregants which were really isolated by the methods mentioned above. As seen in the table, ten out of the 12 possible pheno-types which were expected to come from the combination of white leucineless and yellow lysineless were isolated. Among these, some pheno-types such as white lysineless, white leucine-lysineless, white prototroph, yellow prototroph, green leucineless, green lysineless, and green leucine-lysineless are apparently recombinants.

Moreover, grounding on the number of nuclei in a conidium and the absence of further segre-

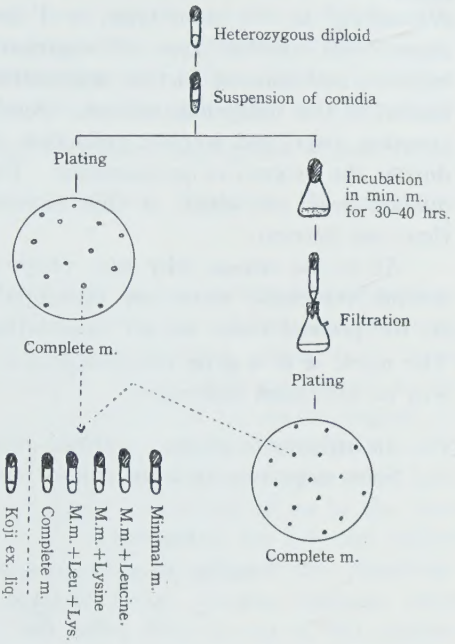


Fig. 4. A Diagram Illustrating the Methods Employed for the Isolation of Prototrophic and Auxotrophic Segregants from a Heterozygous Diploid

Table 2. The First Order Somatic Segregation

Diploid	Segregants		No. of possible geno-type	
	Pheno-types	No. of isolates	Diploid	Haploid
wY leu LYS / Wy LEU lys	White leucineless	25	6	2*
	White lysineless	13	6	2
	White leu-lys-less	13	3	2
	White prototroph	9	12	2
	Green leucineless	12	8	1
	Green lysineless	3	8	1
	Green leu-lys-less	3	4	1
	Green prototroph	(24)**	16	1
	Yellow leucineless	0	4	1
	Yellow lysineless	(83)***	4	1
	Yellow leu-lys-less	0	4	1
	Yellow prototroph	24	8	1

* Notice that *w* is epsiatic to *y* and *Y*.

** The pheno-type of the parental diploid strain is green prototrophic.

*** The yellow colonies were isolated intentionally to look for yellow leucineless and yellow leu-lys-less segregants.

gation, all of the white lysineless strains were proved to be haploids, corresponding to the geno-type; *w Y (or y) LEU lys*. On the other hands, there were another type of segregants which contained less nuclei than haploids and showed further segregation. These are still diploids as be discussed in the following section. Anyhow, it is obvious that the somatic crossing over and somatic reduction in term of Pontecorvo had taken place during the process of propagation. The frequency of crossing over and reduction was not clear in this experiment, but it was supposed to be less than one percent.

As to the reason why two pheno-types, yellow leucineless and yellow leucine-lysineless, were not recovered, we have to consider linkage groups. At the present time, we are postulating three linkage groups; *w y leu lys k*. The mark *k* is a gene responsible for kojic acid production and the detail will be discussed elsewhere.

7. *An attempt to produce triploid and tetraploid strains*

Some experiments were carried out to produce a triploid and a tetraploid

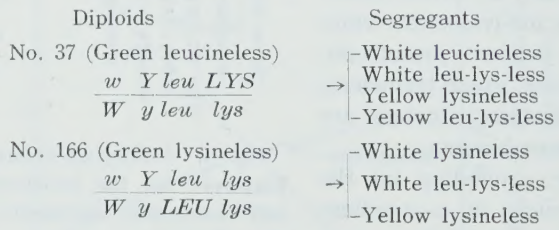


Fig. 5. The Second Order Somatic Segregation

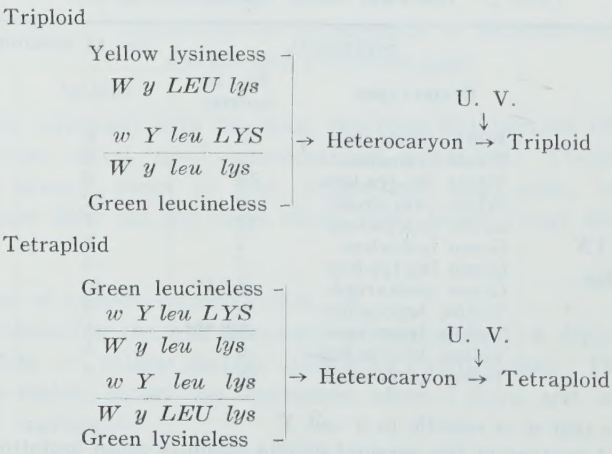


Fig. 6. Production of Triploids and Tetraploids by Ultraviolet Irradiation

from the cross between a diploid and a haploid and between two diploids. The diploids used in these experiments were isolated as first order somatic segregants of a diploid. No. 37, green leucineless, segregated white leucineless, white leucine-lysineless, yellow leucineless and yellow leucine-lysineless as second order somatic segregants; therefore, the geno-type of this strain may be $\frac{w\ Y\ leu\ Lys}{W\ y\ leu\ lys}$. No. 166, green lysineless, segregated white lysineless, white leucine-lysineless, and yellow lysineless; therefore, the geno-type of this strain may be $\frac{w\ Y\ leu\ lys}{W\ y\ LEU\ lys}$.

Crosses were carried out between a yellow lysineless haploid and a green leucineless diploid, and between a green leucineless diploid and a green lysineless diploid. The irradiation of the heterocaryons arising from these mixtures resulted in stable green prototrophs which were considered to be triploid or tetraploid, although the evidence that these are really triploid or tetraploid is not sufficient to present here. In addition to this, it is also regrettable that the increase of polyploidization by means of ultraviolet light s not so clear in the formation of the triploid and tetraploid as in the case of diploid, for the reason that the crosses were carried out between yellow and green and between green and green strains. In spite of this disadvantage, we can say with certainty that at least three possible triploids were obtained out of 563, and at least one possible tetraploid out of 740 colonies which appeared on minimal medium after irradiation. These values are significantly higher than those expected spontaneously.

8. *Effect of ultraviolet light on the segregation of a diploid*

To test the effect of ultraviolet light upon the segregation of a diploid strain, diploid conidia were exposed to ultraviolet light through the conditions mentioned in section 5. Counting the number of sectored colonies among the total survivors at different doses, we have found that the frequency of sectored colonies in *Asp. sojae* increased with irradiation time; the

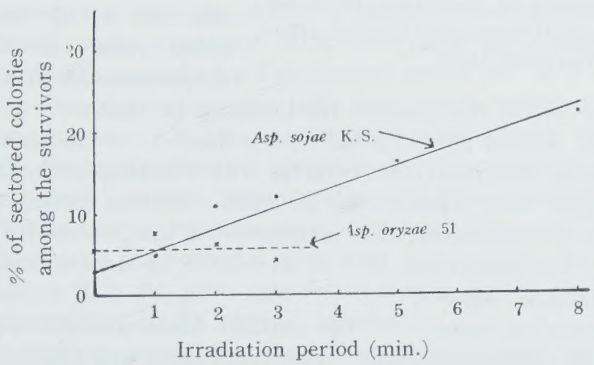


Fig. 7. Effect of Ultraviolet Light on Segregation

values, as shown in Figure 7, 16.2 percent after 5 minutes and 22.5 percent after 8 minutes, are too high to be expected from induced mutation. But in *Asp. oryzae*, a very few sectored colonies appeared after irradiation. From these data, we cannot avoid the conclusion that the effect of ultraviolet light on segregation depends on the strain and is not as uniform as the effect on diploidization which has been reproduced with good agreement in four different strains.

9. *Survival curves of two haploids, the heterocaryon, and the heterozygous diploid*

As an aid to make a speculation about the effect of ultraviolet light upon diploidization, the survival curves of two haploids, the heterocaryon, and the heterozygous diploid were compared. Judging from the data illustrated in Figure 8, it is very hard to think that the diploid conidia have a selective advantage in survival during irradiation.

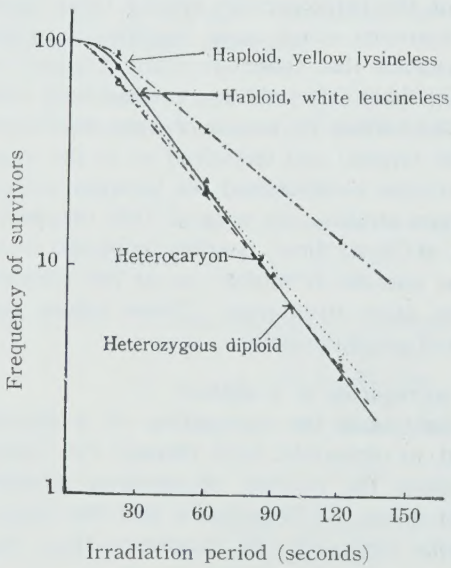


Fig. 8. Frequency of Surviving Haploids, Heterocaryon, and Heterozygous Diploids after Exposure to Ultraviolet Light

DISCUSSION

Since the discovery of somatic recombination in *Asp. nidulans*, several works have been published with other fungi. With regard to the frequency of diploidization, Pontecorvo and his associates have reported that it is about 10^{-7} in *Asp. nidulans*[2], 10^{-7} in *Asp. niger*[4], and 10^{-7} in *Pen. chrysogenum*[5]. The author's data are about 10^{-6} in *Asp. sojae*[8],[9] and less than 5×10^{-4} in *Asp. oryzae*. These values are the frequency of spontaneous diploidization and are too low to utilize this process for breeding purposes.

Therefore, several attempts have been made to promote the frequency through treating heterocaryotic mycelia with chemicals. Among these chemicals, camphor was reported to be most effective for the purpose. For example, some investigators have demonstrated that, through the treatment, the frequency increased from 10^{-7} to $10^{-6}\sim10^{-4}$ in *Asp. nidulans*[2] and from 10^{-7} to 10^{-6} in *Asp. niger*[4].

In the preceding paper[8],[9], the authors have already compared these chemicals with ultraviolet light. The conclusion was that ultraviolet light is thousands of times more effective than the others in case of multinucleate

fungi such as *Asp. oryzae* and *Asp. sojae*. This conclusion was identified again in this paper, though there was no control experiment regarding the chemicals.

Discussion must now turn to the mechanism by which ultraviolet light acts and to the specificity of ultraviolet light action. However, it is very regrettable at the present time that the authors have no special datum and idea as to the mechanism. As seen in Figure 8, the diploid conidia have no selective advantage in survival during irradiation. So, it is possible to postulate a gene mutation, to the direction that favors nuclear fusion between two defferent nuclei, as in the case of relative heterothallism in *Asp. nidulans*[2] and D gene mutation in Saccharomyces yeast[12]. But still, this idea is no more than a speculation.

As to the specific action of ultraviolet light upon different types of strain, the authors have already manifested the same effect with four different combinations of mutants, but extensive tests with another kind of fungi have not yet been finished. In addition, the authors have some data supporting the view that X ray is also effective on diploidization, although the data are not sufficient to draw a conclusive remark. Taking these situations into account, the authors wish to confine their conclusion by saying that ultraviolet light is effective only upon multinuculeate fungi in which heterocaryotic condition can exist in a conidium.

In contrast to the obscurity of the effect of ultraviolet on diploidization, there are some papers which deal with the effect of ultraviolet light on the segregation of a diploid. For instance, Lederberg[13] has reported that ultraviolet light promoted the segregation of a heterozygous diploid of *Escherichia coil* and James[14],[15] has suggested that the somatic recombination-like phenomenon in Saccharomyces yeast increased with the irradiation. Accordingly, this paper can be taken as additional evidence concerning the effect of ultraviolet light on segregation.

In this connection, the authors must give consideration to so-called Lindegren's gene conversion theory[16]. According to Lindegren, in a heterozygous diploid having, for example, *AD*/*ad* structure, the gene *AD* or *ad* becomes labile when it is irradiated with ultraviolet light, resulting in the formation of *AD*/*AD* or *ad*/*ad*. This theory seems to be a critical view contradicting the idea that ultraviolet light does excite segregation through an unknown mechanism. Of this, the authors have already started an investigation with yeast, expecting to elucidate the mechanism more clearly with the comparison of somatic recombination-like phenomenon and the gene conversion. The first paper has been published from Saito and Takahashi[17] of this laboratory, with the use of Lindegren's strain, with the result that another explanation than the gene conversion theory would be possible for the irregular segregation of the adenine genes.

Anyhow, it must be noted that the effect of ultraviolet light upon diploidization (the fiirst effect of U. V.) seems to be a different fact than that

upon segregation (the second effect of U. V.).

SUMMARY

1. The presence of parasexual life cycle in *Aspergillus oryzae* was identified again.

2. The frequency of diploidization was increased from less than 5×10^{-4} to a few percent of the survivors by irradiating heterocaryotic conidia with ultraviolet light. The value is sufficiently high to utilize the process for breeding purpose.

3. Comparing the survival curves of two haploids and the heterocaryon with that of the heterozygous diploid, the diploid conidia were considered to have no selective advantage in survival during the irradiation.

4. As the first order somatic segregants of a diploid, ten out of the twelve possible phenotypes which were expected to come from the combination of white leucineless and yellow lysineless were really recovered. Some of them were proved to be still diploids.

5. A few supposed triploid and tetraploid strains were produced from the cross between a haploid and a diploid and between two diploids.

6. Ultraviolet light was demonstrated to be effective upon the segregation of a diploid as well as on the formation of a diploid. The effect on the former depends on the strain.

7. An attempt which has been carried out to improve industrial strains through this process will be published later.

ACKNOWLEDGEMENT

The authors wish to acknowledge the valuable advice and criticism of Professor Kinichiro Sakaguchi of Tokyo University, Professor Francis J. Ryan of Columbia University, and Dr. G. Sermonti of Istituto Superiore di Sanita in Italy. They are also indebted to Dr. Nobuyoshi Iguchi of Noda Shōyu Co. Ltd. for supplying the mutant strains.

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J. Gen. Appl. Microbiol.
Vol. 3, No. 1, 1957

ON THE CLASSIFICATION OF THE α-KETOGLUTARIC ACID ACCUMULATING BACTERIA IN AEROBIC FERMENTATION

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Received for publication Dec. 15, 1956

INTRODUCTION

Up to the present a number of bacteria which are capable of producing α-ketoglutaric acid during the aerobic fermentation of glucose have been isolated by authors from many soils and sewage and we obtained many bacteria which accumulated a remarkable amount of α-ketoglutaric acid, and particularly among them some produced as much as 40%–60% of the acid of the initia lglucose supplied. Since we were greatly interested in the taxonomic study on these α-ketoglutaric acid producing bacteria, especially on the bacteria which gave such a high yield of 40%–60% of the acid, to glucose consumed, we proceeded the detailed taxonomic study on the selected eleven strains from these bacteria. This report is concerned with the experimental bacteriological description and classification and more especially the proposal of the new genus *Kluyvera*. Some descriptions of only one strain *Bacterium* No. 84C was previously reported[1], and short communication limited on a new genus *Kluyvera* was published in Proceedings of the Japan Academy[2].

In this study the most important description concerns the properties of flagella of these bacteria, one group having peritrichous flagella, and the other group having polar flagella. Furthermore, both groups vigorously ferment glucose, lactose and various sugars with the production of acid and gas. Therefore, in conformity with the classification in Bergey's Manual of Determinative Bacteriology 6th Ed. (1948), it is clear that although the former group should be placed in the family *Enterobacteriaceae*, the latter group should not be assigned to this family by virture of their polar flagella. According to our study it was ascertained that six bacteria belong to the family *Enterobacteriaceae* and more exactly, five bacteria to the genus *Escherichia* and one to the genus *Aerobacter*.

The remained other five bacteria appeared polarly flagellated, gram-negative and non sporogenous rods and ferment vigorously lactose, glucose and various sugars with the production of acid and gas as mentioned above. Therefore, they may be assigned either to the family *Pseudomonadaceae* or to the family *Bacteriaceae*.

The latter family *Bacteriaceae* contains only one genus, *Bacterium*. However, the generic name *Bacterium* Ehrenberg 1828 was rejected by the International Committee on Bacteriological Nomeculature held in Rome in 1953 and this rejection was clearly justified by Buchanan in 1955[3]. And consequently the family name *Bacteriaceae* was also rejected[4].

In 1936 Kluyver and Van Niel[5] published a paper on the porspects for a natural system of classification of bacteria and showed that the generic status in the various tribes may be determined on the basis of catabolic properties. In this classification they suggested that a bacterial group in the tribe *Pseudomonadeae* showing mixed acid fermentation which at the same time unexpectedly corresponded to the genus *Escherichia* in the tribe *Escherichеae*, should be made a separate genus if the existence of such a group of bacteria could be confirmed in future. Our new isolated polar-flagellated bacteria quite clearly coincide in their properties with this bacterial group and should be assigned to this separate genus. Consequently, we propose that a new genus, *Kluyvera*, named in honor of the late Professor A. J. Kluyver, should be formed in the tribe *Pseudomonadeae* and our new five bacteria are to be assigned to the genus *Kluyvera*.

In the following bacteriological descriptions various experimental methods principally referred to "Manual of Methods for Pure Culture Study of Bacteria" published by the Society of American Bacteriologist (1952). Methods and results of morphological, cultural, physiological and special characteristics are described as follows.

MORPHOLOGICAL CHARACTERISTICS

Morphological differences between eleven bacteria usually present only in their flagellation and motility. On bouillon agar they appear as a short rod with round ends measuring 0.7–1.1μ by 1.0–4.0μ, but sometimes filamentous cells as long as 10μ–13μ are observed. They usually occur singly but occasionally in pairs. Motility is examined by hanging drop preparation of young culture on bouillon agar slant. Ten bacteria excepting only one, strain No. 9, show active motility. Gram-stain examined by Hucker modification and furthermore Kopeloff-Beerman modification using one and five days' bouillon agar cultures. They are all gram-negative. Endospores of five days' cultures on bouillon agar were tested with Doner's[6] method but not detected. As flagellation is the most important key in this classification, its determination was precisely investigated and performed with flagella staining of Bailey[7] modification and, for varidity, with the electron microscopic observation. As a result of both examinations five bacteria viz., No. 3, No. 5, No. 7, No. 8 and No. 12, were approved to possess peritrichous flagella and the other five bacteria viz., No. 84C, No. 4, No. 6, No. 10 and No. 11 which should be assigned to the new genus *Kluyvera*, were proved conclusively to possess polar flagella (Table 1).

Table 1. Sources and morphological characteristics.

Strain number	Source	Size of majority (μ)	End
No. 84C	uncultivated soil of Tokyo district	0.7–1.0×1.7–4.0	rounded
3	sewage of Keihin district	0.7–1.0×1.7–4.0	rounded
4	same as above	0.8–1.1×1.7–4.5	rounded
5	same as above	0.6–0.9×1.7–3.0	rounded
6	sewage of Ueno Zoological Garden, Tokyo	0.9–1.0×1.7–3.0	rounded
7	sewage of Kawasaki-shi	0.8–0.9×1.5–3.5	rounded
8	field soil near Kawasaki-shi	0.8–1.0×1.2–3.5	rounded
9	sewage of Keihin district	0.8–1.0×1.5–3.5	rounded
10	same as above	1.0–1.1×1.8–4.7	rounded
11	same as above	0.9–1.1×1.3–3.7	rounded
12	same as above	0.9–1.1×1.4–3.0	rounded

Strain number	Motility	Grame-stain	Endospore	Flagellation
No. 84C	+	−	−	polar
3	+	−	−	peritrichous
4	+	−	−	polar
5	+	−	−	peritrichous
6	+	−	−	polar
7	+	−	−	peritrichous
8	+	−	−	peritrichous
9	−	−	−	none
10	+	−	−	polar
11	+	−	−	polar
12	+	−	−	peritrichous

CULTURAL CHARACTERISTICS

Slight differences of usual cultural characteristics were presented by eleven bacteria. Bouillon agar colonies of these bacteria at 30°C were punctiform, smooth, low convex, entire margin, shiny moist, semi-translucent and grayish white to white and in the mature stage turned to opaque and light yellowish gray to grayish white. In these cultures, media of solid agar were unchanged. On agar stroke growth was abundant and filiform to spreading. In broth clouding of growth was moderate and fluid turbid. Formation of pellicle in liquid culture did not occur, excepting three strains viz., No. 4, No. 8 and No. 10. Results are shown in Table 2. On potato slant growth was abundant, moist shiny, opaque, spreading and gray to light yellowish

gray. Pigment was not produced.

Table 2. Cultural characteristics.

Strain number	Bouillon agar colony	Bouillon agar stroke	Bouillon liquid	
			surface growth	clouding
No. 84C	punctiform, smooth,	abundant,	none	moderate, fluid turbid
3		filiform to	none	slight, fluid turbid
4	low convex, entire margine,	spreading	none	moderate, fluid turbid
5			none	moderate, fluid turbid
6	shiny moist,semi-translucent,		none	moderate, fluid turbid
7	grayish white to white,		slight ring	moderate, fluid turbid
8	and in mature stage turned to opaque, light		thin membrane	strong, fluid turbid
9	yellowish gray to grayish white,		none	almost translucent
10	medium unchanged,		none	moderate, fluid turbid
11			none	moderate, fluid turbid
12			none	moderate, fluid turbid

Strain number	Sediment	Membrane on peptone water
No. 84C	grayish white, compact	none
3	grayish white, compact	none
4	grayish white, compact	thin
5	grayish white, compact	none
6	grayish white, compact	none
7	grayish white, compact	none
8	grayish white, compact	thin
9	grayish white, flocculent	none
10	grayish white, compact	thin
11	grayish white, compact	none
12	grayish white, compact	none

PHYSIOLOGICAL CHARACTERISTICS

1) Relation to free oxygen: eleven bacteria grew well throughout bouillon deep agar medium in a test tube and consequently these bacteria were classified as facultative anaerobes.

2) Temperature relation.

a) Temperature requirement: culture liquid was so called peptone water, namely composed of 1% of polypeptone (Takeda), 0.5% of sodium chloride and the pH was 7.0. Inoculum per 5 ml medium in one test tube was small one drop, approximately 0.05 ml, of 18–24 hrs culture of the above described liquid. Culture was kept for 3 days at 25–27°C, 30°C, 37°C and 42°C. Results are described in Table 3.

Table 3. Temperature relation.

Strain number	Temperature requirement (growth at various temperature)				Thermal death point			
	25–27°C	30°C	37°C	42°C	40°C	50°C	60°C	70°C
No. 84C	+	++	+	−	+	+	−	−
No. 3	+	++	+	−	+	+	−	−
4	+	++	+	−	+	+	−	−
5	+	++	+	−	+	+	−	−
6	+	++	+	−	+	+	−	−
7	+	++	++	+	+	+	−	−
8	++	++	++	+	+	+	−	−
9	+	++	+	−	+	+	−	−
10	+	++	+	−	+	+	−	−
11	+	++	+	−	+	+	−	−
12	+	++	+	−	+	+	−	−

b) Thermal death point: this examination was carried out on heat shock of the same inoculated culture liquid as tested in temperature requirement for 10 minutes at 40°C, 50°C, 60°C, and 70°C. After heat shock, culture liquids were kept at 30°C for 3 days. Results are summarized in Table 3. As in the table, eleven bacteria all grew at 25–37°C and optimum temperature was 30°C. Only two bacteria, No. 7 and No. 8 grew at 42°C, however the other bacteria did not grow at this temperature. Ten minutes at 60°C was judged as thermal death point.

3) Relation to reaction of medium: as a result of the peptone-water culture at following pH; 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0, it was clear that all bacteria grew vigorously at pH 7.0 and at the more acidic side or at the more alkaline side growth became slight. At pH 3.0 and 10.0 growth did not occur.

4) Action on nitrate: investigation of action on nitrate was conducted with nitrate-peptone-water culture containing 0.1% of KNO_3 at 30°C. Nitrite formation from nitrate was tested by using α-naphtylamine-sulfuric acid reagent. Bacterial culture liquids were tested on the 1st, 3rd, 5th and 12th day and in every case nitrite was detected but nitrogen gas did not produced. Consequently all eleven bacteria are capable to reduce nitrate to nitrite.

5) Liquefaction of gelatin: stabs were made in bouillon gelatin and incubated

at 20°C for six weeks. Growth took place merely along the stab line. Only by the strain No. 8 saccate-liquefaction occcurred. The other bacteria did not act on gelatin.

6) Change in litmus milk: skimmed "city" milk was once more boiled, filtered and diluted with three times the volume of distilled water, before adding litmus saturated solution and autoclaved at 115°C for 15 minutes. Incubation was conducted at 30°C. In all bacteria color turned to light red and gas produced within 3 days but coagulation made slow progress. Peptonization was not observed. Results are described in Table 4.

Table 4. Change in litmus milk.

Strain number	3rd day		7th day		11th day		18th day	
	color	coagulation	color	coagulation	color	coagulation	color	coagulation
No. 84C	R	–	R	+	R	+	R	+
No. 3	R	–	R	–	R	+	R	+
4	R	–	R	–	R	+	R	+
5	R	–	R	–	R	+	R	+
6	R	–	R	+	R	+	R	+
7	R	–	R	–	R	–	R	–
8	R	–	R	–	R	–	R	–
9	R	–	R	±	R	+	R	+
10	R	–	R	–	R	+	R	+
11	R	–	R	–	R	–	R	+
12	R	–	R	+	R	+	R	+

R: Red

7) Methyl red test and Voges-Proskauer test: a culture medium composed of 0.5% of glucose, 0.5% of polypeptone and 0.5% of K_2HPO_4 was incubated at 30°C for 7 days. Methyl red test was prepared with addition of methyl red alcohol solution and Voges-Proskauer test with addition of 5% α-naphtol alcohol solution and 40% KOH solution. Both tests were conducted with 3, 5 and 7 day's culture liquids respectively. As the results of these tests, the eleven bacteria showed an inverse correlation between methyl red test and Voges-Proskauer test. Results are as in the following.

MR	VP	Strain
+	–	No. 84C, No. 3, No. 4, No. 5, No. 6, No. 7, No. 9, No. 10, No. 11, No. 12.
–	+	No. 8.

8) Utilization of citrate as a sole source of carbon: Koser's citrate medium

was suitable for this determination and was prepared as follows; 0.1% KH_2PO_4, 0.02% $MgSO_4$, 0.15% $Na_2NH_4PO_4$ and 0.3% $Na_3C_6H_5O_7$. Growth, as evidenced by turbidity, in the clear culture medium indicated utilization of citrate as a carbon source. We made certain that a small enough inoculum was used in this medium so that it was not noticeably turbid before incubation. Bacteria which utilize citrate as carbon source did grow vigorously within 24 hrs but the other which do not utilize citrate showed no growth during 30 days longer. Results are as in the following.

Utilization of citrate	Strain
+	No. 84C, No. 5, No. 6, No. 8, No. 9, No. 11, No. 12.
−	No. 3, No. 4, No. 7, No. 10

9) Production of hydrogen sulfide: two methods, test strip technique and peptone iron agar method, were used. In the former method lead acetate paper was projected into the cotton-plugged test tube, then it turned black in contact with hydrogen sulfide produced from culture medium of peptone water containing 0.05% of cysteine hydrogen chloride or none. Peptone iron agar method (peptone iron agar medium: peptone 2.0%, K_2HPO_4 0.1%, Ferric citrate 0.05%, agar 1.5%.) was exactly referred to the direction of Levine and Vaughn[8] (1934). Cultures were kept at 30°C during 20 days. Results are described in Table 5.

Table 5. Production of hydrogen sulfide.

Strain number	Test strip technique				Peptone iron agar
	0.05% cysteine		none		
	1 day	4 days	1 day	4 days	20 days
No. 84C	++	++	+	++	−
No. 3	++	++	+	++	−
4	+	++	±	+	−
5	++	++	+	++	−
6	++	++	+	++	−
7	++	++	±	++	−
8	++	++	±	++	−
9	++	++	−	±	−
10	++	++	−	++	−
11	−	++	−	++	−
12	++	++	+	++	−
E. coli	+	++	−	±	−
E. freundii					+

These results show that the production of hydrogen sulfide could be detected in all bacteria when using the test strip technique, which is very sensitive, but could not be detected when using an insensitive method such

as the peptone iron agar method. Previously Hunter and Weiss[9] (1938) reported that practically all members of the *coli-aerogenes* group produced hydrogen sulfide when tested with a sensitive indicator and only differences in the quantity of hydrogen sulfide produced were presented between these organisms. However the production of hydrogen sulfide is an important key in distinguishing species in the genus *Escherichia*, it seemed most proper that all eleven bacteria tested were regarded as positive in production of hydrogen sulfide.

10) Hydrolysis of Starch: on the bouillon agar containing 0.2% of soluble starch test organisms were smeared and incubated at 30°C for 14 days. On 2nd, 4th and 14th day Lugol's solution was dropped on to each culture plate but it remained blue. Consequently starch was not hydrolyzed.

11) Indole and ammonia production: indole and ammonia production from peptone water cultures at 30°C were detected by the use of Kovács[10] reagent for indole and Nessler's reagent for ammonia. Results are as in the following.

Indole	Ammonia	Strain
+	+	No. 84C, No. 7, No. 9, No. 11, No. 12.
−	+	No. 3, No. 4, No. 5, No.. 6, No. 8. No. 10.

12) Fermentation of carbohydrates: culture medium employed for the fermentation of carbohydrates was peptone water in test tube containing 0.5% of various carbohydrates and brom cresol purple as the indicator and was putted in with small Durham tube. Production of acid was detected by color change of culture liquid, purple to yellow, and gas production was observed

Table 6. Fermentation of carbohydrates.

Source	Arabinose	Rhamnose	Xylose				Glucose	Fructose	Galactose
Days / Strain number	3	3	3	6	12	30	3	3	3
No. 84C	AG	AG	AG				AG	AG	AG
3	AG	AG	AG				AG	AG	AG
4	AG	AG	−	−	−	−	AG	AG	AG
5	AG	AG	AG				AG	AG	AG
6	AG	AG	AG				AG	AG	AG
7	AG	AG	AG				AG	AG	AG
8	AG	AG	AG				AG	AG	AG
9	AG	AG	AG				AG	AG	AG
10	AG	AG	−	−	−	−	AG	AG	AG
11	AG	AG	AG				AG	AG	AG
12	AG	AG	AG				AG	AG	AG

Table 6. (Continued).

Source	Mannose	Lactose		Sucrose				Maltose	Trehalose	Melibiose
Days / Strain number	3	3	6	3	6	12	30	3	3	3
No. 84C	AG	AG		AG				AG	AG	AG
3	AG	AG		AG				AG	AG	AG
4	AG	AG		AG				AG	AG	AG
5	AG	AG		—	—	—	—	AG	AG	AG
6	AG	AG		AG				AG	AG	AG
7	AG	AG		AG				AG	AG	AG
8	AG	—	AG	AG				AG	AG	AG
9	AG	AG		AG				AG	AG	AG
10	AG	AG		AG				AG	AG	AG
11	AG	AG		AG				AG	AG	AG
12	AG	AG		—	AG			AG	AG	AG

Source	Cellobiose				Raffinose				Melezitose				Starch				Inulin			
Days / Strain number	3	6	12	30	3	6	12	30	3	6	12	30	3	6	12	30	3	6	12	30
No. 84C	AG				AG				—	—	—	—	—	—	—	—	—	—	—	—
3	AG				AG				—	—	—	—	—	—	—	—	—	—		
4	AG				AG				—	—	—	—	—	—	—	—	—	—	—	—
5	AG				—	—	—	AG	—	—	—	—	—	—	—	—	—	—	—	—
6	AG				AG				—	—	—	—	—	—	—	—	—	—	—	—
7	—	—	—	—	AG				—	—	—	—	—	—	—	—	—	—		
8	AG				AG				—	—	—	—	—	—	—					
9	AG				AG				—	—	—	—	—	—	—	—	—	—	—	
10	AG				AG				—	—	—	—	—	—	—	—	—	—	—	—
11	AG				AG				—	—	—	—	—	—	—	—	—	—	—	
12	AG				AG				—	—	—	—	—	—	—	—	—	—	—	

AG: Acid and gas

with the elevation of gas in Durham tube. Cultures were kept statically at 30°C and followed until 30th day. During this period observation was carried on and results are listed in Table 6.

As are shown in these results, all eleven bacteria generally ferment vigorously monosaccharides and disaccharides with the production of acid and gas. Exceptionally only two bacteria, No. 4 and No. 10 do not ferment xylose. All bacteria ferment lactose and glucose with acid and gas production and it is much interested that polarly flagellated five bacteria ferment lactose vigorously as glucose with acid and gas production. Starch, Inulin, Melezitose and some sugar alcohols are not fermented.

Table 6. (Continued).

Source	Dextrin				Glycogen				Glycerol				Erythritol				Mannitol
Strain number \ Days	3	6	12	30	3	6	12	30	3	6	12	30	3	6	12	30	3
No. 84C	—	G			—	—	—	—	G	AG			—	—	—	—	AG
3	—	G			—	—	—	—	AG				—	—	—	—	AG
4	—	G			—	—	—	—	—	AG			—	—	—	—	AG
5	—	G			—	—	—	—	AG				—	—	—	—	AG
6	—	G			—	—	—	—	—	—	—	—	—	—	—	—	AG
7	—	—	—	—	—	—	—	—	—	AG			—	—	—	—	AG
8	—	—	—	—	—	—	—	—	—	—	—	G	—	—	—	—	AG
9	—	G			—	—	—	—	—	—	AG		—	—	—	—	AG
10	—	G			—	—	—	—	—	AG			—	—	—	—	AG
11	—	—	G		—	—	—	—	—	—	—	—	—	—	—	—	AG
12	—	G			—	—	—	—	—	AG			—	—	—	—	AG

Source	Sorbitol				Dulcitol				Aesculin
Strain number \ Days	3	6	12	30	3	6	12	30	3
No. 84C	—	—	—	—	—	—	—	—	AG
3	—	—	—	—	—	—	—	—	AG
4	—	—	—	—	—	—	—	—	AG
5	AG				—	—	—	—	AG
6	AG				AG				AG
7	AG				—	AG			AG
8	AG				—	—	—	—	AG
9	—	—	—	—	—	—	—	—	AG
10	—	—	—	—	—	—	—	—	AG
11	AG				—	—	—	—	AG
12	—	—	—	—	—	—	—	—	AG

G: gas only.

13) Catalase reaction: catalase reaction was recognized to be positive in all bacteria in accordance with the production of numerous foams when the young cells grown on agar slant were suspended into 3% hydrogen peroxide solution.

14) Utilization of uric acid as the sole source of nitrogen: Koser[11] (1918), Chen and Rettger[12] (1920) and many subsequent workers found that, whereas *aerogenes* strains are uric acid positive, strains of *coli* and intermediate type are uric acid negative. This test is important key and mentioned in descriptions of the genus *Escherichia*. From these reasons we

adoped this test. Determination of utilization of uric acid is exactly same as the utilization of citric acid. Koser's[11] synthetic medium was used: 0.5% of NaCl, 0.02% of $MgSO_4 \cdot 7H_2O$ 0.01% of $CaCl_2 \cdot 2H_2O$ 0.1% of K_2HPO_4, 3% of glycerol and 0.05% of uric acid in ammonium free water. Culture was kept at 30°C for 7 days. Growth, evidenced by turbidity, assures the utilization of uric acid as the sole source of nitrogen. Results are as in the following.

Utilization of uric acid	Strain
+	No. 8.
−	No. 84C, No. 3, No. 4, No. 5, No. 6, No. 7, No. 9, No. 10, No. 11, No. 12.

Only strain No. 8 could utilize uric acid as the sole source of nitrogen.

15) Ratio H_2:CO_2 of gas produced from glucose in liquid medium: when the fermentation of 0.5% glucose contained in peptone-water at 30°C finished after 72 hrs in the Smith's fermentation tube, volume of gas produced in this tube was determined. Next, into the fermented liquid 4% KOH solution was added and CO_2 included in produced gas was absorbed into the alkaline fermented liquid. The volume of residual gas was measured. Furthermore, as the explosion of hydrogen with oxygen was brought out with a roar when burning the residual gas in flame in contact with air, this residual gas was assumed to be hydrogen. These results are described in Table 7. This

Table 7. Ratio H_2:CO_2 of gas produced from glucose.

Strain number	Total gas (ml)	H_2 (ml)	CO_2 (ml)	H_2/CO_2
No. 84C	4.10	2.90	1.20	2.4
No. 3	2.70	2.10	0.60	3.5
4	1.00	0.80	0.20	4.0
5	3.15	2.20	0.95	2.3
6	3.30	2.30	1.00	2.3
7	—	—	—	—
8	5.00	2.00	3.20	0.63
9	3.50	2.60	0.80	3.3
10	3.40	3.10	0.80	3.9
11	4.30	3.00	1.50	2.0
12	—	—	—	—

method referred to Exercise No. 39 of "An Introduction to Laboratory Technique in Bacteriology 3rd Edition" published by The MacMillan Company in New York.

16) Urease test: when a lump of young cells grown on agar slant was incubated in the urea-phenol red solution, if this organism possesses urease,

the solution turns to red. Our eleven bacteria were incubated in this solution for 60 minutes but urease could not be detected by this method, although *Proteus vulgaris* turned the solution to red in these minutes.

17) Utilization of metabolic intermediates of glucose as the sole source of carbon: procedures and culture conditions were almost identical with those in the utilization of citrate. Various metabolic intermediates of glucose described in Table 8 were used as the sole source of carbon which took the place of citrate in Koser's citrate medium. Cultures were followed at 30°C for 20 days. Utilization of these compounds was evidenced by growth. Gluconate, 2-ketogluconate, pyruvate, lactate, malate and oxalacetate were

Table 8. Utilization of metabolic intermediates of glucose as the sole source of carbon.

Carbon source	Gluconic				2-ketogluconic				Pyruvic				Lactic			
Strain number \ Days	1	5	10	20	1	5	10	20	1	5	10	20	1	5	10	20
No. 84C	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+
3	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+
4	+	+	+	+	−	+	+	+	+	+	+	+	±	+	+	+
5	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+
6	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+
7	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+
8	+	+	+	+	±	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	−	+	+	+	+	+	+	+	±	+	+	+
10	+	+	+	+	−	+	+	+	+	+	+	+	±	+	+	+
11	+	+	+	+	−	+	+	+	+	+	+	+	±	+	+	+
12	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+

Carbon source	Acetic				Citric				α-ketoglutaric				Succinic			
Strain number \ Days	1	5	10	20	1	5	10	20	1	5	10	20	1	5	10	20
No. 84C	−	±	+	+	+	+	+	+	−	−	−	−	−	+	+	+
3	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
4	−	−	+	+	−	−	−	−	−	−	−	−	−	±	+	+
5	−	−	±	+	+	+	+	+	−	−	−	−	−	−	−	±
6	−	−	+	+	+	+	+	+	−	−	−	−	−	−	+	+
7	−	−	±	+	−	−	−	−	−	+	+	+	−	+	+	+
8	−	+	+	+	+	+	+	+	−	+	+	+	±	+	+	+
9	−	−	±	+	+	+	+	+	−	−	−	−	−	−	±	+
10	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	+
11	−	−	±	+	+	+	+	+	−	−	−	−	−	−	−	+
11	−	−	+	+	+	+	+	+	−	−	−	−	−	−	+	+

Table 8. (Continued).

Carbon source	Fumaric				Malic				Oxalacetic			
Days / Strain number	1	5	10	20	1	5	10	20	1	5	10	20
No. 84C	−	+	+	+	+	+	+	+	+	+	+	+
3	−	−	−	−	+	+	+	+	+	+	+	+
4	−	−	±	+	+	+	+	+	+	+	+	
5	−	−	−	+	+	+	+	+	+	+	+	
6	−	−	+	+	+	+	+	+	+	+	+	
7	−	+	+	+	+	+	+	+	+	+		
8	+	+	+	+	+	+	+	+	+	+	+	
9	−	−	+	+	+	+	+	+	+	+	+	
10	−	−	±	+	+	+						
11	−	−	−	+	+	+	+	+	+	+	+	
12	−	−	+	+	+	+	+	+	+	+		

easily utilized as the sole source of carbon by eleven bacteria, and acetate, succinate and fumarate not easily utilized and α-ketoglutarate was not utilized by the nine bacteria excepting the strain No. 7 and No. 8. Only strain No. 8 was seen to utilize many intermediates easily. It is of great interest and possibly rather reasonable that nine bacteria which accumulate a remarkable amount of α-ketoglutaric acid in fermented liquid could not utilize this product as the sole source of carbon. Results are shown in Table 8.

18) Chromogenesis and fluorescence: in rather long periodic culture on solid surface such as bouillon agar, alkali peptone agar, glucose ammonium salts agar and potato slant at 20–30°C, chromogenesis was not observed. However only in the aerobic α-ketoglutaric acid fermentation of glucose light yellow color was produced in the broth and when the culture was prolonged, turned dark brown. During the fermentation feeble fluorescence was produced.

19) Action of coli-phage: coli phage T_1, T_2, T_4 and T_7 were examined on five polarly flagellated bacteria respectively, however actions were all negative.

Classification of eleven bacteria according to Bergey's Manual of Determinative Bacteriology 6th Edition (1948).

I) Non-polarly flagellated bacteria.

In this group six bacteria viz., No. 3, No. 5, No. 7, No. 8, No. 9 and No. 12 are included. They are short, peritrichous flagellated, gram-negative and non-sporogenous rods, however only No. 9 exceptionally non-motile with no flagellum, and all reduce nitrate to nitrite and are active in fermentation of sugars, especially they ferment glucose and lactose with the production of acid and gas. From these characteristics these bacteria are classified to the

family *Enterobacteriaceae* Rahn. They ferment actively various sugars with the production of acid and gas, grow well in ordinary media, have a feeble proteolytic activity, possess no urease, and don't show remarkable chromogenesis in ordinary media. Consequently it is most proper that these bacteria should be assigned to the tribe *Eschericheae* Bergey, Breed and Murray. In the present Bergey's classification the tribe *Eschericheae* contains three genera such as *Escherichia*, *Aerobacter* and *Klebsiella*. The genus *Klebsiella* is distinguished from other two genera according to the formation of capsule in the mucoid phase. However, as our six bacteria have no capsule and don't show any mucosity, they should belong to the genus *Escherichia* or *Aerobacter*.

Key to the genera of tribe *Eschericheae*

I. Acetylmethylcarbinol not produced. Methyl red test positive.
Salts of citric acid may or may not be used as a sole source of carbon.
Genus I *Escherichia*

II Acetylmethylcarbinol produced. Methyl red test negative.
Salts of citric acid used as sole source of carbon.
Genus II *Aerobacter*.

According to this key assignment of six bacteria is to be as following.

genus *Escherichia*: Strain No. 3, No. 5, No. 7, No. 9, and No. 12.
genus *Aerobacter*: Strain No. 8

Key to the Species of Genus *Escherichia* Castellani and Chalmers.

I. Citric acid and salts of citric acid not utilized as sole source of carbon.
A. Hydrogen sulfide not produced. 1. *Escherichia coli*.

II. Citric acid and salts of citric acid utilized as sole source of carbon.
A. Hydrogen sulfide produced. 2. *Escherichia freundii*
B. Hydrogen sulfide not produced. 3. *Escherichia intermedium*.

On the basis of previous described characteristics five bacteria of the genus *Escherichia*, Strain No. 3, No. 5, No. 7, No. 9 and No. 12 are to be classified as in the following.

Escherichia coli citrate — H_2S — Strain No. 3 and No. 7,
Escherichia freundii 〃 + 〃 + Strain No. 5, No. 9 and No. 12.

Key to the species of genus *Aerobacter* Beijerinck.

I. Glycerol fermented with acid and gas.
A. Gelatin not liquefied (rarely liquefied) 1. *Aerobacter aerogenes*

II. Glycerol fermented with no visible gas.
A. Gelatin liquefied. 2. *Aerobacter cloacae*.

Strain No. 8, as previously decribed, ferments glycerol, feebly liquefies gelatin, and clearly it should be assigned to *Aerobacter cloacae*.

II) Polarly flagellated bacteria.

This grouped bacteria viz., strain No. 84C, No. 4, No. 6, No. 10 and No.11 are all free living, non-sporogenous, gram-negative and polarly flagellated short rods, and according to these characteristics they should clearly be assigned to the family *Pseudomonadaceae* Winslow et al. and furthermore to the tribe *Pseudomonadeae* Kluyver and Van Niel. On determination of assigned genus in the tribe *Pseudomonadeae* they are closely related to the genus *Pseudomonas* rather than four genera, *Xanthomonas*. *Methanomonas*, *Acetobacter* and *Protaminobacter*. However it is certainly inappropriate to assign them to the genus *Pseudomonas* because they have some distinctive characteristics different from this genus such as being active in fermentation of sugars, especially lactose, with production of acid and gas, rather they minic the bacteria of the genus *Escherichia* in many bacteriological characteristics excepting fllagella.

Pseudomonas fermentans Kühr[13] 1932 is polarly flagellated rods and ferments strongly various sugars, also lactose, forming acid and gas. In 1936 Kluyver and Van Niel[6] proposed that this species should be assigned to the separate genus from the genus *Pseudomonas* and this separate genus should be called *Aeromonas*. However *Pseudomonas fermentans* is certainly different from our new group of bacteria because it carries out 2, 3-butylenglycol fermentation, methyl red test is negative and inversely the Voges-Proskauer test is positive.

It was proposed to assign *Pseudomonas lindnerii* (Kluyver and Hoppenbrouwers[14] 1931), which carries out an alcoholic fermentation, to the separate genus, *Zymomonas*. But this is also clearly distinguished from our polarly flagellated bacteria.

From these discussions on classification of our new group of bacteria, we came to the following conclusion: it is inappropriate to assign them to the genus *Pseudomonas* or *Zymomonas* and it is reasonable to assign them to a new separate genus.

Assignment to the genus *Bacterium* can not be discussed because this generic name was rejected as previously described at the beginning of this paper.

In 1936 Kluyver and Van Niel published a paper on bacterial classification and proposed two genera in the tribe *Pseudomonadeae*, one genus performing mixed acid fermentation and the other genus carrying out 2, 3-butylenglycol and acetion fermentation. Exactly the same differentiation is made in the tribe *Escherichеae*, the former type being assigned to the genus *Escherichiae* and the latter type to the genus *Aerobacter*.

To the latter genus performing 2, 3-butylenglycol and action fermentation, the generic name *Aeromonas* was presented and described in Kluyver's classification, however the species assigned to the former genus was not isolated and necessarily this genus was vacant. Of course they anticipated the pre-

sence of this bacterial group which is polarly flagellated and performs mixed acid fermentation.

In 1943 Hitchner[15] suggested the group of acid and gas-producing pseudomonads should be rearranged in a separate genus, *Aeromonas*. However as the generic name *Aeromonas* had been proposed previously by Kluyver and Van Niel, this genus should be limited to a group of bacteria which carriss but 2, 3-butylenglycol and action fermentation.

For these reasons, we reached the conclusion that a new genus, *Kluyvera*, should be founded in the tribe *Pseudomonadeae* being separated from *Aeromonas*, and thus our new five bacteria, No. 84C, No. 4, No. 6, No. 10 and No. 11 can be included in this new genus. The description of the genus is as follows.

In this description Bacterium No. 84C corresponds to *Kluyvera citrophila* var. α, No. 6 to *Kluyvera citrophila* var. β, No. 11 to *Kluyvera citrophila* var. γ, and No. 4 and No. 10 to *Kluyvera noncitrophia*.

Genus *Kluyvera* gen. nov. Asai

Named for the late Prof. A. J. Kluyver, who first suggested the existence of this type of organism.

Gram-negative short rods. Motile with polar flagella. Ferment glucose and lactose with acid and gas (hydrogen present). Nitrites are produced from nitrates. Acetylmethylcarbinol is not produced. Methyl red test is posive. Milk is usually coagulated, but peptonization does not occur. Gelatin is not liquefied. Grow well on ordinary media and pigment is not produced. Found in soil and sewage.

The type species is *Kluyvera citrophila* Asai.

Key to the species of genus *Kluyvera*

I. Citric acid and salts of citric acid utilized as sole source of carbon.

1. *Kluyvera citrophila*

II. Citric acid and salts of citric acid not utilized as sole source of carbon.

2. *Kluyvera noncitrophila*

1. *Kluyvera citrophila* sp. nov. Asai

From Latin *citrus*, the citron tree; M. L., lemon or orange, hence citric acid, and Greek *philus*, loving.

Short rods: 0.7 to 1.0 by 1.0 to 5.0 microns. Usually single, occasionally in pairs. Longer filamentous irregular forms are often seen. No capsule or endospore observed. Motile, with a single, or occasionally two or three polar flagella. Gram-negative.

Gelatin colonies: Small, punctiform, grayish white, smooth, moist, glistening, flat. Transparent, becoming opaque. Edge is entire to undulate. No liquefaction.

Gelatin stab: Uniform surface growth. Line of puncture is filiform. No

liquefaction.

Agar colonies: Small (as in gelatin colonies) to large, incomplete, circular colonies. Other characteristics are nearly the same as gelatin colonies.

Agar slant: Abundant growth. White, filiform to spreading, glistening. Medium unchanged. Non-chromogenic.

Broth: No pellicle. Moderate turbidity with compact, gray sediment. Amount of sediment is moderate.

Production of indole and fermentation of carbon sources by varieties of *Kluyvera citrophila*

	α	β	γ		α	β	γ
Production of indole	+	−	+	Fermentation of cellobiose	AG	AG	AG
Fermentation of				raffinose	AG	AG	AG
arabinose	AG	AG	AG	melezitose	—	—	—
xylose	AG	AG	AG	starch	—	—	—
rhamnose	AG	AG	AG	dextrin	G	G	G
glucose	AG	AG	AG	inulin	—	—	—
fructose	AG	AG	AG	glycogen	—	—	—
galactose	AG	AG	AG	glycerol	AG	AG	—
mannose	AG	AG	AG	erythritol	—	—	—
lactose	AG	AG	AG	mannitol	AG	AG	AG
sucrose	AG	—	AG	sorbitol	—	AG	AG
maltose	AG	AG	AG	dulcitol	—	AG	—
trehalose	AG	AG	AG	esculin	AG	AG	AG
melibiose	AG	AG	AG	salicin	AG	AG	AG

AG: Acid and gas G: Gas only

Litmus milk: Red with acid production. Coagulum is formed. No peptonization.

Potato: Abundant growth. Grayish, moist.

Nitrates reduced to nitrites.

Ammonia produced.

Starch not hydrolyzed.

H_2S produced..

Methyl red test positive. Voges-Proskauer test negative.

Citrate utilized.

α-Ketoglutarate not utilized.

Uric acid not utilized.

Catalase positive.

Urease negative.

See the above table for production of indole and fermentation of carbon sources.

Ratio of H_2 to CO_2 produced is 2.0 to 2.4 in 72 hours' culture in a 0.5%

glucose peptone water (measured in Smith's fermentation tube).

Fecal to fragrant oder produced in shaken culture.

Anaerobic, facultative.

Temperature relations: Optimum 30°C. No growth at 42°C.

Death point: 60°C for 10 minutes.

pH range: Optimum pH 7.0. No growth at pH 3.0 or 10.0.

Chromogenesis: Usually grayish-white to light yellowish gray white on ordinary media. No remarkable pigment produced. In shaken culture, yellow to dark yellow pigment and feeble fluorescence produced.

Source: From soil and sewage.

Type strain (*Kluyvera citrophila* α) has been deposited in the Japanese Federation of Culture Collections of Microorganisms.

Note: The authors recognize three varieties of this species which are differentiated on their ability to ferment carbon sources and to produce indole. It is noteworthy that all varieties are capable of producing a large amount of α-ketoglutaric acid from glucose in shaken culture. Variety α was isolated from soil and was reported as *Bacterium* strain No. 84C(1) (temporary name) in 1955. Varieties β and γ were isolated from sewage.

2. *Kluyvera noncitrophila* sp. nov. Asai

Short rods: 0.7 to 1.0 by 1.0 to 4.5 microns. Usually single, occasionally in pairs. Longer filamentous irregular forms are often seen. No capsule or endospore observed. Motile, with a single, or occasionally two or three polar flagella. Gram-negative.

Gelatin colonies: Same as *Kluyvera citrophila*.

Gelatin stab: Same as *Kluyvera citrophila*.

Agar colonies: Same as *Kluyvera citrophila*.

Agar slant: Same as *Kluyvera citrophila*.

Broth: Same as *Kluyvera citrophila*.

Peptone-water: Thin film, easily broken by mild shaking.

Litmus milk: Same as *Kluyvera citrophia*.

Potato: Abundant growth. Grayish to light yellowith gray, moist.

Indole not formed.

Nitrates reduced to nitrites.

Ammonia produced.

Starch not hydrolyzed.

H_2S produced.

Methyl red test positive. Voges-Proskauer test negative.

Citrate not utilized.

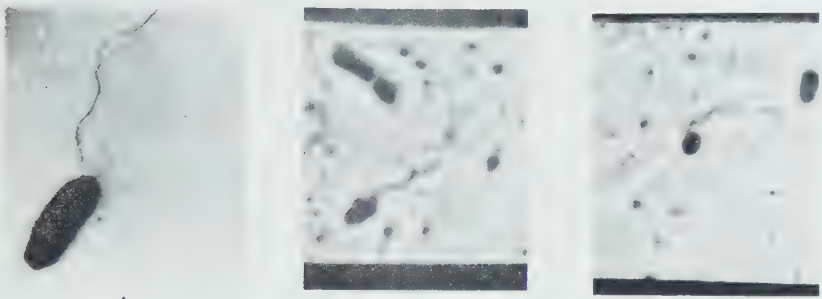
α-Ketoglutarate not utilized.

Uric acid not utilized.

Catalase positive.

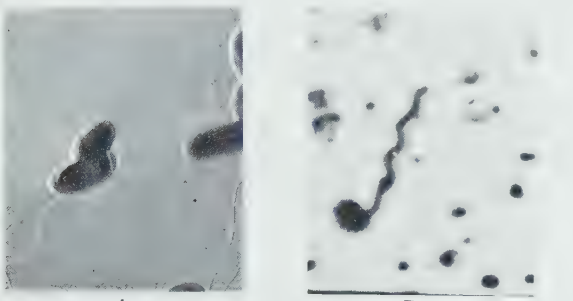
Urease negative.

Acid and gas from arabinose, rhamnose, glucose, fructose, galactose,



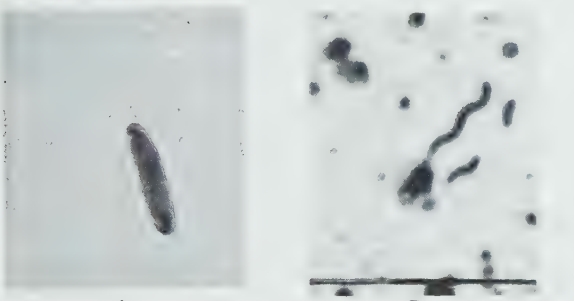
A B C

Fig. 1. *Kluyvera citrophila* α:
A, Electronmicrograph ×10000. B, C, Bailey flagella stain. Photomicrograph ×2200.



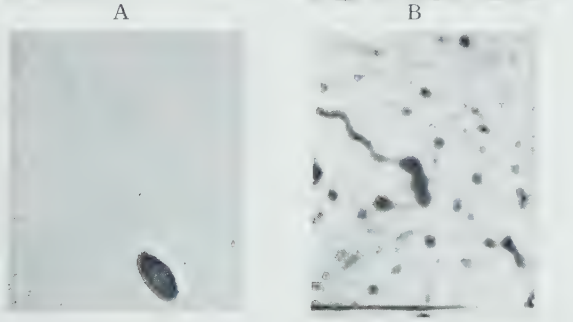
A B

Fig. 2. *Kluyvera citrophila* β:
A, Electronmicrograph ×5200.
B, Bailey flagella stain. Photomicrograph ×2200.



A B

Fig. 3. *Kluyvera citrophila* γ:
A, Electronmicrograph ×5200.
B, Bailey flagella stain. Photomibrograph ×2200.



A B

Fig. 4. *Kluyvera noncitrophila*:
A, Electronmicrograph ×5200.
B, Bailey flagella stain. Photomicrograph ×2200.

Note: Leifson flagella strain noted in a short communication published in Proceedings of the Japan Academy[2] is corrected as Bailey flagella stain.

mannose, lactose, sucrose, maltose, trehalose, melibiose, cellobiose, raffinose, glycerol mannitol, esculin, and salicin.

Some gas from dextrin.

No acid or gas from xylose, melezitose, inulin, starch, glycogen, erithritol, sorbitol and dulcitol.

Ratio of H_2 to CO_2 produced is 4.0 in 72 hours' culture in a 0.5% glucose peptone water (measured in Smith's fermentation tube).

Fecal to fragrant oder produced in shaken culture.

Anaerobic, facultative.

Temperature relations: Optimum 30°C. No growth at 42°C.

Death point: 60°C for 10 minutes.

pH range: Optimum pH 7.0. No growth at pH 3.0 or 10.0.

Chromogenesis: Same as *Kluyvera citrophila*.

Source: From sewage.

Type strain has been deposited in the Japanese Federation of Culture Collections of Microorganisms.

Note: This species accumulates a large amount of α-ketoglutaric acid, formed from glucose in shaken culture.

Discussion on some bacteria to be rearranged in the genus *Kluyvera*.

In our opinion, at least three bacteria viz., *Bacterium ketoglutaricum*[16] (Masuo 1955), *Pseudomonaas coli communis*[17] (Conn, Esten and Stocking 1956) and *Pseudomonas formicans*[18] (Crawford 1954) should be rearranged in the genus *Kluyvera*.

Bacterium ketoglutarium possesses a single polar flagellum and carries out the mixed acid fermentation.

Pseudomonas coli communis was described insignificantly in Bergey's manual of Determinative Bacteriology 6th Ed. 1948, however according to its polar flagella and being similar to *Escherichia coli*, assignment to the genus *Kluyvera* is possibly considered.

SUMMARY

Eleven bacteria, which were newly isolated from soils and sewage and are capable of producing α-ketoglutaric acid with higher yields such as 40–60% to glucose supplied in aerobic fermentation, were studied from the taxonomic point of view. Precise descriptions of morphological, cultural and physiological characteristics were performed. As a result of these descriptions two bacteria were identified as *Escherichia coli*, three as *Escherichia freundii*, one as *Aerobacter cloacae*. However the remaining five bacteria possessed polar flagella and fermented glucose, lactose and other sugars vigorously with the production of acid and gas. After exhaustive discussion we reached the conclusion that they should be assigned to a new genus in the tribe *Pseudomonadeae*. Consequently we proposed a new genus *Kluyvera* to which

they should be assigned. A description of the new genus and descriptions of two new species of the genus *Kluyvera* are presented.

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J. Gen. Appl. Microbiol.
Vol. 3, No. 1, 1957

FERMENTATIVE PRODUCTION OF L-GLUTAMIC ACID FROM α-KETOGLUTARIC ACID AND AMMONIUM SALT*

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Received for publication Dec. 20, 1956

INTRODUCTION

Up to the present, L-glutamic acid has been produced by the hydrolysis of protein materials such as soybean or wheat, or by the cleavage of pyrrolidone carboxylic acid contained in beet molasses. In these procedures the yield of L-glutamic acid is dependent on the content of L-glutamic acid in the materials. On the other hand, when the glutamic acid was chemically synthesized the process of separating L-glutamic acid from D-glutamic acid is needed. Such separation processes are cumbersome and expensive.

Recently, Johnson[1] pointed out that there is, in many quarters, great interest in fermentative glutamic acid production. Masuo *et al*[2] observed the presence of transaminase activity between alanine and α-ketoglutaric acid in *Bacterium ketoglutaricum* which can produce α-ketoglutaric acid from glucose, and they succeeded in producing L-glutamic acid from glucose by adding alanine in the later stage of the culture. Though it requires other amino acids as the donor of the amino group, this method may be highly appreciated as one of the promising procedures for fermentative production of L-glutamic acid.

More recently, Smythe *et al*[3] devised an enzymatic method. They use homogenate of various cells or tissues which have the enzymatic activity of L-glutamic dehydrogenase. To this homogenate α-ketoglutaric acid and ammonium are added with a hydrogen donor which serves to reduce the oxidized coenzyme of the dehydrogenase.

The use of living cells for L-glutamic acid formation is more applicable for industrial purpose. Morrison and Hinshelwood[4], and Morrison *et al*[5] reported that the trace of L-glutamic acid was formed in the culture of *Escherichia coli* or *Aerobacter aerogenes*. In their experiments synthetic medium containing glucose and an ammonium salt was used.

The activity to accumulate L-glutamic acid in the medium was, however, so low that the application of these results to industrial production of L-glutamic acid is not possible.

* This work was presented at the meeting of The Institute for Food Technology Aichi Prefecture, held on August 1, 1956.

The formation of amino nitrogen from ammonium and keto acids using suspensions of *Bacillus subtilis*, *Cholera vibrio* and *Escherichia coli*, and the formation of L-glutamic acid are also reported by Konikova *et al*[6),(7)].

The present study was carried out to search for the microorganisms which do accumulate L-glutamic acid in the medium containing α-ketoglutaric acid and an ammonium salt, and to investigate the cultural conditions which are in favor of the accumulating L-glutamic acid.

Some works on the mechanism of L-glutamic acid formation are also presented.

EXPERIMENTAL

(I) Isolation of the microorganisms

The composition of the media used for the isolation of L-glutamic acid forming strains were as follows;

Medium A_1	Na-α-ketoglutarate	1.0% (as free acid)
	glucose	0.1
	NH_4Cl	0.5
	K_2HPO_4	0.1
	$MgSO_4 \cdot 7H_2O$	0.05
Medium A_2	medium A_1+Casein-tryptic-hydrolyzate 0.01%	
Medium A_3	medium A_1+Casein-tryptic-hydrolyzate 0.008%+Yeast extract 0.007%	

In each case the pH was adjusted to 7.0 and 1:1 mixture of distilled water and tap water was used.

Earlier, this experiment was carried out using the medium A_1, the simplest of these three media. Later the medium A_2 and also the medium A_3 were used, because there must be strains which can accumulate L-glutamic acid but can not grow on such a simple medium as medium A_1.

Microorganisms were isolated from a variety of soil samples. About 0.1 g of the soil was suspended in 5 ml of the sterilized water and the suspension was plated out on the agar media.

After 48 hrs'. incubation at 25, 30 and 37°C, the organisms grown on the agar plates were transferred to the test tubes containing 5 ml of liquid media.

The stationary test tube cultures were also incubated at the three temperatures, 25, 30 and 37°C. After 5 days' incubation, the cultures were centrifuged to remove the cells and the supernatants were used for the determination of L-glutamic acid. The determination of L-glutamic acid was made by use of paper chromatogram. The solvent used was a mixture of n-buthanol 4, acetic acid 1, and water 5. After development, the paper-chromatograms were dried and sprayed with 0.1% ninhydrin solution. The colored spots located at the position corresponding to L-glutamic acid were compared with that of the standard solution of L-glutamic acid and the concentrations of L-glutamic acid were roughly estimated.

The results of the screening test for L-glutamic acid production were summurized in Table 1. Among the 1599 strains isolated, only 62 strains

Table 1. Summary of the Screening Test for L-Glutamic Acid (L-glu.) Production by Bacteria Isolated from Soil.

Activity to accumulate L-glu.	Numbers of the isolated strains	Ratio
##	62	3.9%
+ or ±	364	22.7
−	1173	73.4
Total	1599	100

* Activity to accumulate L-glu. is indicated with the signs representing an amount of accumulated L-glu. after 5 days incubation as follows;

##: $> 5\,\mu$M/ml

+: $5\,\mu$M/ml $>$, $> 1\,\mu$M/ml

produce L-glutamic acid with a concentration higher than $5\,\mu$M per ml of culture media.

The accumulation of L-glutamic acid was observed in the cultures of the microorganisms belonging to the following genera;

Bacillus, *Xanthomonas*, *Pseudomonas*, *Micrococcus*, *Aerobacter* and *Aeromonas*.

The strains belonging to the Genus *Pseudomonas* were isolated more frequently than the others, and were proved to have a strong tendency to accumulate L-glutamic acid.

Moreover, more than one hundred strains of type cultures supplied by The Institute of Applied Microbiology, The University of Tokyo, were tested for their ability to accumulate L-glutamic acid. It was found that in addition to the above listed genera the organisms belonging to the following genera were also able to accumulate L-glutamic acid in the medium A_3;

Bacterium, *Agrobacterium*, *Erwinia*, *Serratia*, *Escherichia*, *Debaryomyces*, *Hansenula*, *Mycotorula*, *Pseudosaccharomyces*, *Sacchromyces*, *Willia*, *Aspergillus*, *Penicillium* and *Rhizopus*.

(II) On the cultural conditions for L-glutamic acid accumulation by *Pseudomonas ovalis* No. 259–2

Strain, Methods and Chemicals.

Strain; A newly isolated strain No. 259–2 which showed the highest level of L-glutamic acid accumulation and was found to belong to *Pseudomonas ovalis*, was used in the following experiments. The characteristics of *Pseudomonas ovalis* No. 259–2 are as follows.

Description of the properties of Pseudomonas strain No. 259–2

Pseudomonas strain No. 259–2 was isolated in 1955 from an uncultivated soil in the Tokyo district.

Cells are medium sized rod:
0.6 to 0.8 by 1.5 to 2.4 microns. Actively motile with a polar flagellum. Gram negative.

Nutrient agar colony:
Circular, entire, raised to convex, smooth to verrucose, brownish gray, butyrous.

Nutrient agar slant:
Aboundant growth, filiform, glistening, brownish gray, medium becoming greenish yellow.

Nutrient broth:
Pellicle, strong fluid turbid, sediment moderate, flocculent.

Nutrient gelatine:
Best growth at top, forming greenish fluorescent pigment, not liquefaction.

Litmus milk:
Alkaline; not peptonized, reduced and coagulated.

Nitrate not reduced to nitrite.

Indol not formed.

Hydrogen sulfide not formed.

Methyl-red test negative.

Voges-Proskauer test negative.

Citrate assimilated.

Starch not hydrolyzed.

Acid from xylose and glucose without visible gas, and not from glycerin, lactose, sucrose and starch in synthetic medium; and not formed acid and gas from glycerin, xylose, lactose, sucrose and starch in peptone medium.

Assimilated gluconate, citrate, succinate. acetate, lactate, α-ketoglutarate, malonate, p-hydroxybenzoate, glucose, glycerin, ethanol and asparagine.

Optimum temperature:
30°C, poor growth at 37°C.

Aerobic, strict.

Methods; The cultural broth was centrifuged in order to remove the cells. The supernatant was heated in the boiling water for 5 mins., and the L-glutamic acid content of the heated suprenatant was manometrically determined using L-glutamic acid decarboxylase from *Escherichia coli* Crooks[8]. The concentration of α-ketoglutaric acid was determined by the colorimetric method described by Shimizu[9] or by the iodometric titration[10].

Chemicals; Sodium α-ketoglutarate was kindly supplied by the Ajinomoto Co., Inc. L-Amino acids used were commercial products.

(1) *Consumption of α-ketoglutaric acid and accumulation of* L-*glutamic*

acid during stationary culture of Pseudomonas ovalis in the medium A_1.

The active cells of *Pseudomonas ovalis* No. 259–2, taken from the pre-culture in medium A_1 were inoculated in the 500 ml flask containing 300 ml of medium A_1, and were incubated at 30°C. In order to know the activity of this microorganism in accumulating L-glutamic acid, the amount of L-glutamic acid and α-ketoglutaric acid were determined daily during 7 days.

The results are shown in Table 2. The amount of L-glutamic acid in-

Table 2. The Consumption of α-Ketoglutraric Acid and the Accumulation of L-Glutamic Acid by *Pseudomonas ovalis* No. 259–2.

Days	α-Ketoglutaric acid		L-Glutamic acid	
	Residual	Consumed (1)	Accumulated (2)	Yield (2)/(1)
	μM/ml	μM/ml	μM/ml	%
0	74.4	0	0	0
1	65.3	9.1	0.7	7.7
2	38.6	35.8	4.2	11.7
3	22.2	52.1	8.0	15.4
4	6.1	68.3	6.6	9.7
7	3.5	70.9	3.3	4.7

creased in 3 days. The highest accumulation of L-glutamic acid was obtained after 3 days' incubation. The consumption of once accumulated L-glutamic acid was observed after 4 days. The activity to accumulate L-glutamic acid seemed to be high enough to carry out the subsequent experiments.

(2) *Effects of nitrogen sources on L-glutamic acid accumulation.*

The seven nitrogen sources, ammonium chloride, ammonium sulphate, ammonium nitrate, ammonium phosphate, ammonium acetate, ammonium citrate and urea, were tested. Each of them was added to the medium A_3 instead of ammonium chloride with a concentration of nitrogen corresponding that of the medium A_3. Five ml of medium in the test tube was inoculated with the active cells of *Pseudomonas ovalis* No. 259–2. In order to minimize the fluctuation between samples, ten test tubes were prepared for one medium and the cultural broths were randomly sampled from two of them and mixed prior to the determination of L-glutamic acid and α-ketoglutaric acid.

The results are shown in Fig. 1. It would be apparent that ammonium chloride is the best nitrogen source for L-glutamic acid accumulation by *Pseudomonas ovalis* No. 259–2.

(3) *Effects of temperature and oxygen on the accumulation of L-glutamic acid by Pseudomonas ovalis No. 259–2.*

The tests of the influence of incubation temperature and oxygen were designed as follows;

1. The 100 ml Erlenmeyer flask containing 80 ml of medium A_3 was incubated at 30°C.

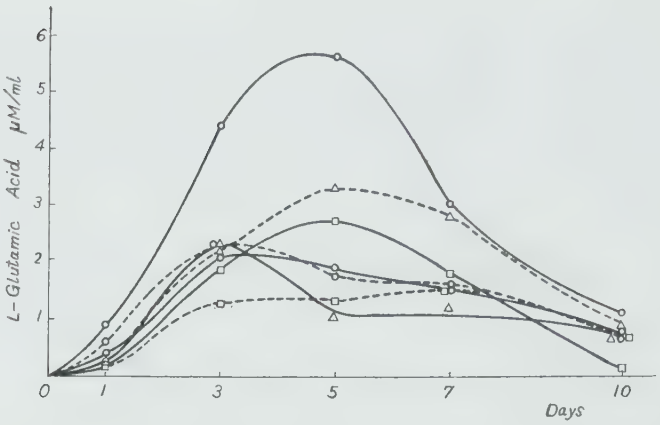

Fig. 1. Effects of Nitrogen Sources on the Accumulation of L-Glutamic Acid by *Pseudomonas ovalis* No. 259-2.
Incubation: At 30°C.
Concentration of N sources: 0.13% as N in the medium.
●——● NH_4Cl ○——○ $(NH_4)_2SO_4$ △——△ NH_4NO_3
□——□ $(NH_4)_2HPO_4$ ○- - -○ NH_4-acetate △- - -△ $(NH_4)_2$-citrate
□- - -□ Urea

2. The same flask was incubated at 25°C.
3. The same flask was incubated at 37°C.
4. The same flask was incubated *in vacuo* at 30°C.
5. The Fernbach flask, with a diameter of 12 cm at the bottom and containing 160 ml medium A_3 was incubated at 30°C.

Each flask was inoculated with 2 drops of the seed culture of *Pseudomonas ovalis* No. 259-2 which was grown in the medium A_3 on the shaker for 17 to 18 hrs. at 30°C.

During the incubation for 10 days, the growth of the cells was estimated turbidimetrically by the optical density at 630 mμ and the amount of L-glutamic acid produced was determined manometrically using a preparation of L-glutamic acid decarboxylase from *E. coli* cells.

The results are shown in Fig. 2. In the stationary Erlenmeyer flask culture at 30°C, the cell growth reached to the stationary phase after 3 to 5 days, showing the highest level of L-glutamic acid accumulation. Although the stationary culture at lower temperatures such as 25°C could support a fairly good growth of the cells, the amount of L-glutamic acid was less than that accumulated at 30°C. When incubated at 37°C, both the growth of cells and the accumulation of L-glutamic acid were poor. On the other hand, the cultures in Fernbach flasks showed a rapid growth and only after 24 hrs. a considerable amount of L-glutamic acid was already found. However, such a large amount of L-glutamic acid as found in Erlenmeyer flask cultures was never accumulated in the medium and the amount of L-glutamic acid

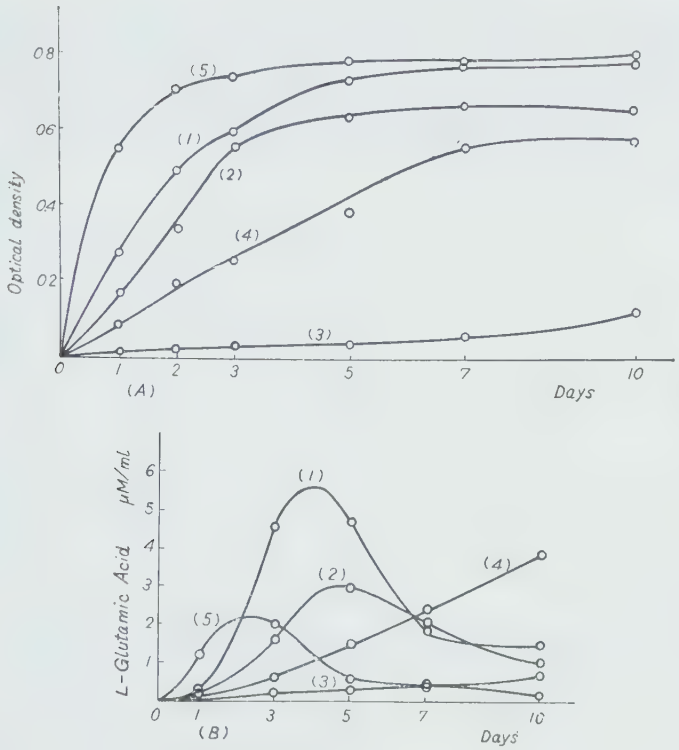

Fig. 2. Effects of Temperature and Oxygen on the Growth of *Pseudomonas ovalis* No. 259–2 (A) and on the Accumulation of L-Glutamic Acid (B).
Medium: A_3.
Incubation: (1) at 30°C, (2) at 25°C, (3) at 37°C, (4) in vacuo at 30°C and (5) Fernbach flask, at 30°C. The growth of the cells was estimated turbidimetrically by the optical density at 630 mμ.

began to decrease after 2 or 3 days. Under the anaerobic condition both the growth of the organism and the accumulation of L-glutamic acid were slow, whereas the amount of L-glutamic acid showed a gradual increase up to 10 days incubation. When this organism was grown in the medium A_3 with constant shaking at 30°C, the cells grew very rapidly. Within 17 to 18 hrs. the optical density of the culture at 630 mμ attained to 0.832 and almost all the α-ketoglutaric acid disappeared showing no accumulation of L-glutamic acid.

These facts suggested that the aerobic conditions were favorable for the growth of *Pseudomonas ovalis* No. 259–2, but, when there were enough cells in the medium, more L-glutamic acid would be accumulated under the anaerobic conditions than that under the aerobic conditions.

In order to examine the above presumption, a test of L-glutamic acid accumulation by a heavy cell suspension was carried out. After *Pseudomonas*

ovalis No. 259–2 was grown in the medium A_3 with constant shaking at 30°C for 17 to 18 hrs., the cells were harvested by centrifugation and washed thrice with physiological saline. The washed cells were added to the fresh medium A_3. The concentration of cell suspension was 30 mg cells (wet weight) per ml. The conditions of incubation were the same as those used in the prior experiments.

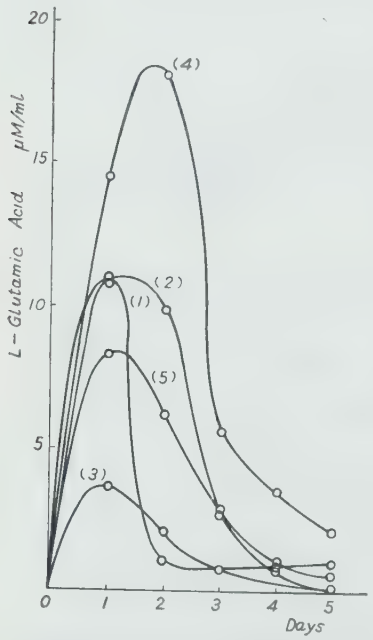

Fig. 3. Effects of Temperature and Oxygen on the Accumulation of L-Glutamic Acid by Heavy Cell Suspensions of *Pseudomonas ovalis* No. 259–2.

Initial concentration of the suspended cells was 30 mg cells (wet weight) per ml of medium A_3.

Incubation: (1) at 30°C, (2) at 25°C, (3) at 37°C, (4) *in vacuo* at 30°C and (5) Fernbach flask, at 30°C.

The results are shown in Fig. 3. It would be noted that within only 24 to 48 hrs., a large amount of L-glutamic acid was accumulated in the suspension, especially when incubated anaerobically at 30°C. The pH of the medium incubated anaerobically remained at 6.8 whereas the pH of the medium incubated aerobically raised to 8 within 24 hrs. It seemed necessary therefore, to pursue the processes of L-glutamic acid accumulation in more detail and to examine the effects of pH on the L-glutamic acid accumulation.

(4) *Effect of the pH on the* L-*glutamic acid accumulation by cell suspension.*

The washed cells prepared as described above were suspended in 80 ml of the fresh medium A_3 in the 100 ml Erlenmeyer flasks. The initial concentration of the cells in the suspension was 30 mg cells (wet weight) per ml.

The pH of the medium was controlled as follows;

1. Initial pH 5.6, and the pH of the medium was adjusted with NaOH or HCl to the initial pH at the intervals of 6 hrs.
2. Initial pH 5.6, and the pH was not adjusted.
3. Phosphate buffer pH 7.4, was added to the medium A_3 to give the concentration of M/30. When the pH change of the buffered medium occurred, the pH of the medium was adjusted at the intervals of 6 hrs.
4. Initial pH 7.2, and the pH of the medium was not adjusted.
5. Initial pH 8.6, and the pH of the medium was adjusted to the initial pH at the intervals of 6 hrs.

All those experiments were conducted both aerobically and anaerobically,

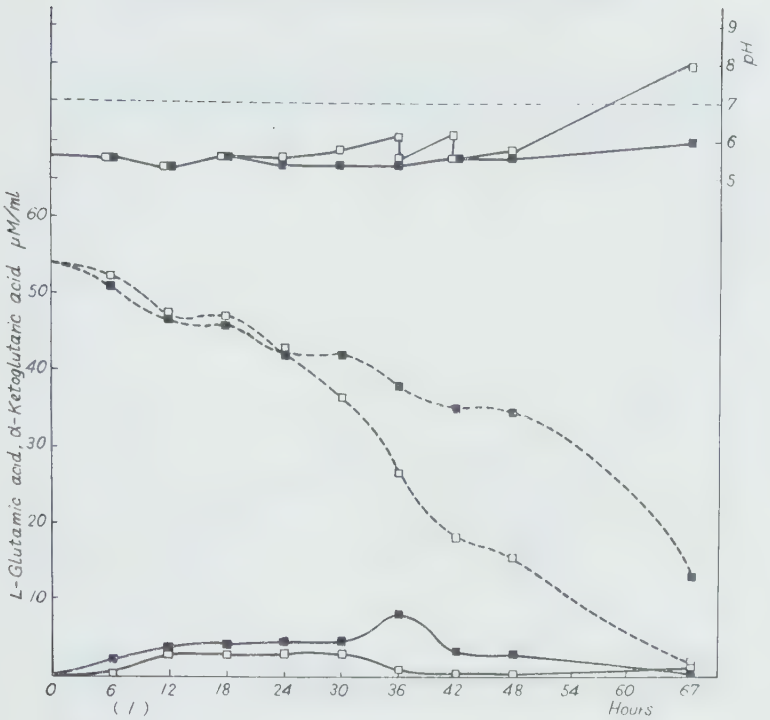
L-Glutamic acid, α-Ketoglutaric acid μM/ml
pH
Hours
(1)

Fig. 4

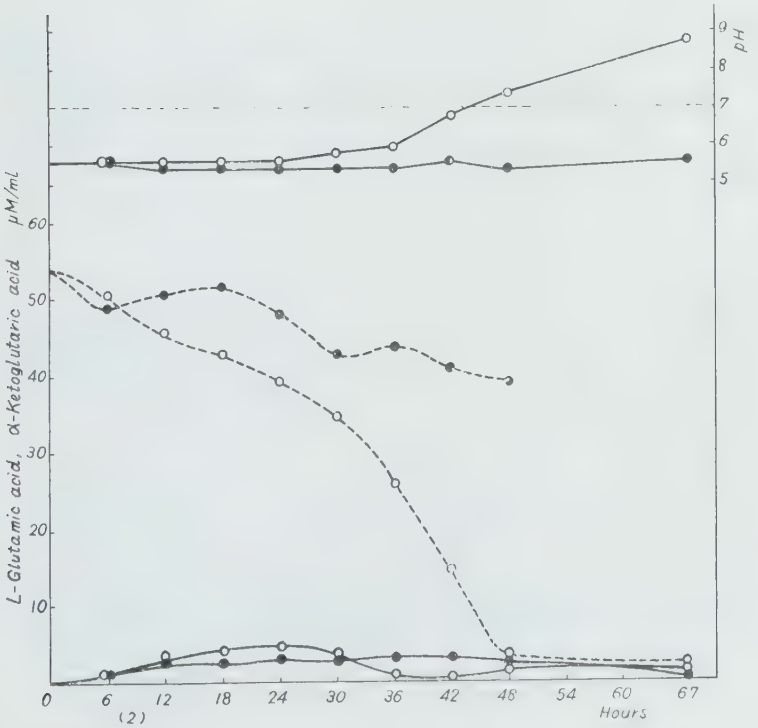
L-Glutamic acid, α-Ketoglutaric acid μM/ml
pH
Hours
(2)

Fig. 4

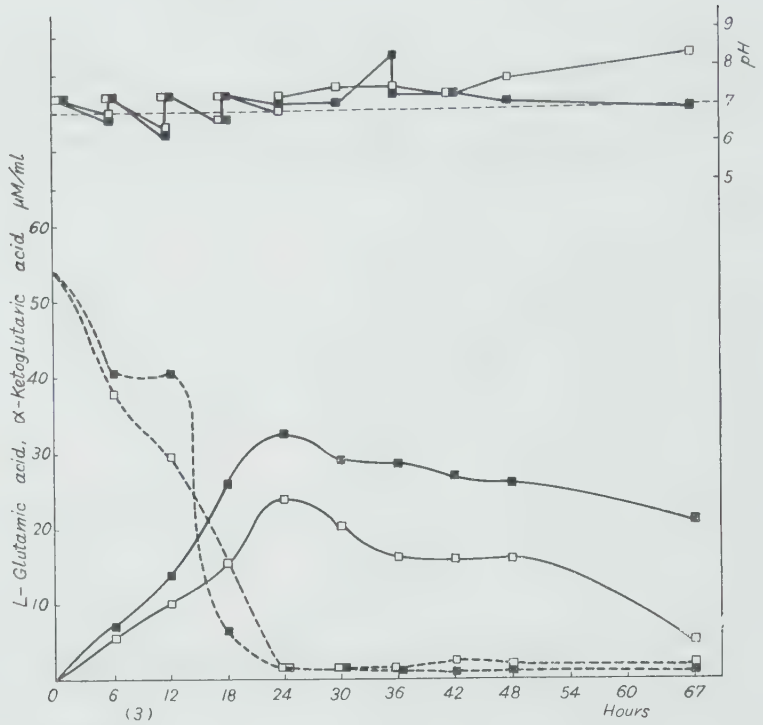
L-Glutamic acid, α-Ketoglutaric acid μM/ml
pH
Hours
(3)

Fig. 4

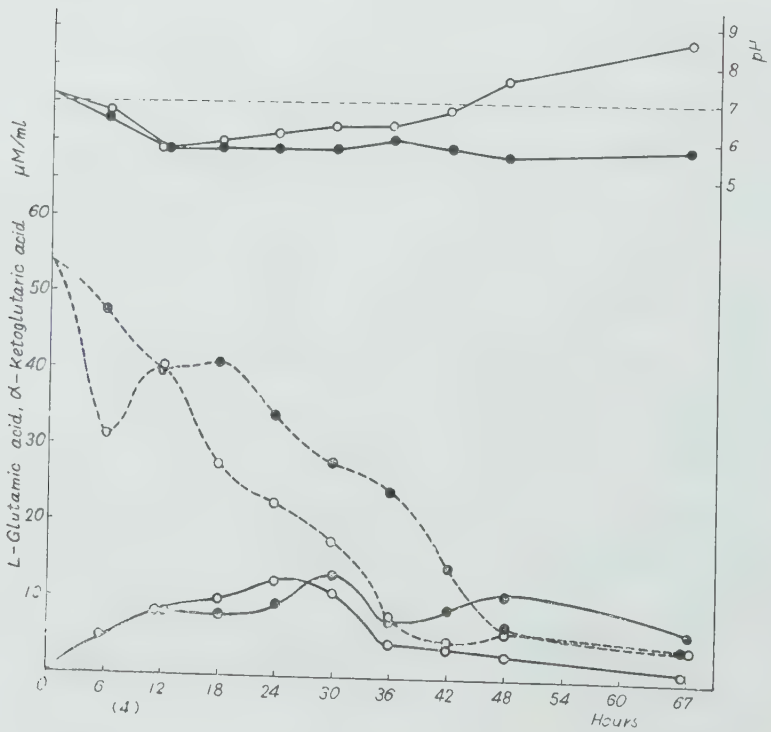
L-Glutamic acid, α-Ketoglutaric acid
μM/ml
pH
Hours
(4)

Fig 4

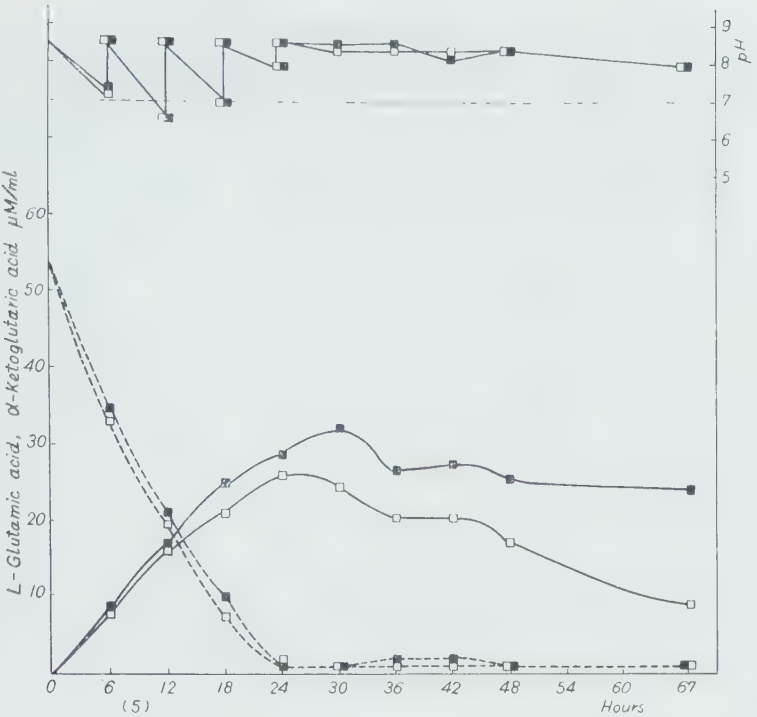

Fig. 4

Fig. 4. Effect of pH on the Accumulation of L-Glutamic Acid by Heavy Cell Suspensions of *Pseudomonas ovalis* No. 259-2.

Initial concentration of the suspended cells was 30 mg cells (wet weight) per ml of medium A_3.

Incubation: At 30°C.

Initial pH: pH 5.6....(1) and (2).
pH 7.4....(3).
pH 7.2....(4).
pH 8.6....(5).

Solid line: L-Glutamic acid.

Dotted line: α-Ketoglutaric acid.

□: Aerobic, pH adjusted.

■: Anaerobic, pH adjusted.

○: Aerobic, pH not adjusted.

●: Anaerobic, pH not adjusted.

and were incubated at 30°C.

Sampling and pH adjustment were carried out at the intervals of 6 hrs. for the 48 hrs. incubation. The incubation after 48 hrs. until 67 hrs. was continued without adjusting pH.

The results are shown in Fig. 4. It is obviously seen that the neutral or slightly alkaline pH is favorable for L-glutamic acid accumulation. An

anaerobic condition was also desirable. The highest accumulation of L-glutamic acid was, therefore, observed when the incubation was carried out anaerobically maintaining the pH at neutral or slightly alkaline.

Within 24 to 30 hrs., about 98% of the added α-ketoglutaric acid was consumed and 30 μM L-glutamic acid per ml, an amount corresponding to 60% of the consumed α-ketoglutaric acid, were accumulated in the medium. On the other hand, under aerobic condition, such a high accumulation of L-glutamic acid was not observed even when pH was maintained neutral or slightly alkaline. When the pH was not controlled, even though it started at pH 7, the amount of L-glutamic acid accumulated was only 1/3 that of the best condition. When the initial pH was acidic, only a very small amount of L-glutamic acid was accumulated. From these results, the effect of pH was proved to be one of the most important factors for the accumulation of L-glutamic acid in the medium. As shown in Fig. 4, the media showed a characteristic changes in pH during the incubation depending on the conditions of incubation.

In the earlier period of incubation, the pH of the medium, which was initially neutral or alkaline, tended to fall down. For example, when the pH started at 7.2, it went down to pH 5.8 within 12 hrs. After 30 to 36 hrs., the pH of the medium under aerobic condition began to increase, and at the end of 67 hrs. incubation it reached to pH 8. In contrast to the aerobic incubation, the medium incubated anaerobically did not show such an increase in pH.

It was confirmed that products of autolysis of bacterial cells could not account for the amount of L-glutamic acid accumulated in the suspension, because no L-glutamic acid was detected in the cell suspension when incubated without α-ketoglutaric acid and ammonium chloride.

(II) Some enzymological investigations using crude extract of cells of *Pseudomonas ovalis* No. 259-2

In order to clarify the mechanism of L-glutamic acid accumulation, some enzymological investigations were carried out. Cells were obtained from shake culture by the same procedures as described in the foregoing experiments. One part of cells was ground in a mortal with two parts of alumina, then extracted with 5 parts of phosphate buffer for an hour. Grinding and extraction were carried out at 0°C. Clear supernatant, *i.e.* crude extract, was obtained by centrifugation, and was stored at −20°C.

(1) *The L-glutamic dehydrogenase activity of the crude extract.*

L-Glutamic dehydrogenase activity was tested by measuring the reduction time of the methylen blue in the Thunberg tubes at 30°C. Protocols of the reaction mixture and results are shown in Table 3.

It was clearly observed that dehydrogenation of L-glutamic acid was markedly accelerated by the triphosphopyridine nucleotide (TPN). This re-

Table 3. L-Glutamic Dehydrogenase Activity of *Pseudomonas ovalis* Cells Demonstrated by Thunberg Method.

Contents of Thunberg tube \ Experimental No.	1	2	3	4	5	6	7	8
0.1 M Na-L-Glutamate	0.2 ml	0.2		0.2	0.2	0.2		
1/10⁴ Methylene blue	1	1	1	1	1	1	1	1
Crude extract *1	1		1					
Dialysed extact *2				1	1	1	1	1
D. P. N. *3					0.2		0.2	
T. P. N. *4						0.2		0.2
Phosphate buffer pH 7.6	4	5	5.2	4	3.8	3.8	3.8	3.8
Time in minutes required for decolorization of methylene blue	36	>120	>120	100	63	23	>120	>120

Thunberg tubes were incubated at 30°C.

*1: Cells were extracted with phosphate buffer pH 7.6.

*2: Dialysed extract was prepared by dialysing the crude extract against the runing tap water for 20 hrs. at 4°C.

*3: D. P. N. was purchased from Mann Research Laboratories, (purity 95%) and was used as water solution (0.5 mg/ml).

*4: T. P. N. was kindly supplied by Mr. S. Morita, (purity 80%) and was used as water solution (0.5 mg/ml).

sults agreed with Euler's observation[11],[12],[13].

(2) *The aspartase activity of the crude extract.*

Reaction mixtures composed of 1 ml of crude extract (extracted with phosphate buffer pH 7.6), 1 ml of 0.2 M fumaric acid (as neutral salt), 1 ml of 0.2 M ammonium chloride and 2 ml of M/15 phosphate buffer (pH 7.6). The mixture was incubated in the evacuated Thunberg tube at 30°C for an hour. Then the tube was holded in the boiling water for 5 mins. to stop the reaction. The precipitated protein was removed by the centrifugation. L-Aspartic acid formed in the reaction mixture was detected by means of paper chromatography. The method of paper chromatography was similar to that used in the screening of the microorganisms. Paper chromatograms showed that aspartic acid was formed when the crude extract was incubated with fumaric acid and ammonium chloride. No amino acids were formed when the heated enzyme preparation was used or when one of the substrates was omitted from the reaction mixture. Microbioassay of L-aspartic acid revealed that 3.7 μM per ml of L-aspartic acid were formed in the reaction mixture.

(3) *The transaminase activity of the crude extract.*

Reaction mixtures composed of 0.5 ml of crude extract (extracted with phosphate buffer pH 7.74), 0.6 ml of 0.2 M L-aspartic acid (as neutral salt), 0.6 ml of 0.2 M α-ketoglutaric acid and 1.5 ml of M/15 phosphate buffer (pH 7.74). The mixtures were incubated in an evacuated Thunberg tube at 30°C

for an hour, then, the reaction was stopped by holding the tube in boiling water for 5mins. Following the addition of 0.8ml of 3M acetate buffer (pH 4.8), the precipitated protein was removed by the centrifugation. The amount of L-glutamic acid formed by the transamination was estimated by the amounts of CO_2 gas liberated by the L-glutamic acid decarboxylase prepared from *E. coli* cells. In the calculation of L-glutamic acid, the amount of CO_2 liberated from the control system (heated enzyme preparation was used) was subtracted from that obtained in the complete system. It was found that 9.28 μM per ml of L-glutamic acid were formed in the reaction mixtures by transamination.

(IV) The effects of hydrogen-donating substances on L-glutamic acid accumulation by *Pseudomonas ovalis* No. 259-2

The enzymological investigations suggested that L-glutamic acid was formed through either one or both, of the following pathways;

1. α-ketoglutarate + $TPNH_2$ + NH_3 —— L-glutamic dehydrogenase ⟶ L-glutamate + TPN + H_2O
2. α-ketoglutarate —— TCA cycle ⟶ fumarate
   fumarate + NH_3 —— aspartase ⟶ L-aspartate
   L-aspartate + α-ketoglutarate —— aspartic-α-ketoglutaric transaminase ⟶ oxalacetate + L-glutamate

When L-glutamic acid was formed through the former pathway, it is necessary to couple the reductive amination of α-ketoglutaric acid with the oxidation of the hydrogen-donating reactant. It is known that α-ketoglutaric acid itself can serve as the hydrogen-donating reactant[14]. It may be more economical, however, to supply with some of the other efficient hydrogen-donating reactants if they work more efficiently. Substances which seemed to be suitable for this purpose was examined. To the cell suspensions prepared as described above a variety of substrates (listed in Table 4) were added together with α-ketoglutaric acid and ammonium chloride. The suspensions using the medium which contained only α-ketoglutaric acid, ammonium chloride and hydrogen-donating reactant was prepared and incubated simultaneously. Incubation was carried out anaerobically for 24hrs. at 30°C and the pH of the medium was adjusted to the initial pH (the initial pH was 7.2) at the intervals of 6 hrs.

As shown in Table 4, no definitely positive effects on the yield and rate of formation of L-glutamic acid were found by the addition of various kinds of substrates. Glucose was rather inhibitory to the formation of the L-glutamic acid. It may be noted here that the larger amount of L-glutamic acid was accumulated in the medium containing only α-ketoglutaric acid and ammonium chloride than that in the medium A_3, from which glucose was omitted, containing the inorganic salts and growth factors additionally. It seemed probable that in the former medium, the normal metabolism in the

Table 4. Effects of the Addition of Some Substrates to the Basal Media on the Accumulation of L-Glutamic Acid by *Pseudomonas ovalis* Cells.

Substrates added	Basal media *1	Concentration of added substrates μM/ml *2	Accumulation of L-glutamic acid	
			Amounts μM/ml	Yield % *3
None (α-ketoglutaric acid alone)	A	—	18.2	49.1
	B	—	29.6	53.5
Formic acid	A	17.5	13.5	37.9
	B	7	23.2	42.9
Acetic acid	A	7	23.6	57.6
	B	7	38.9	67.7
Lactic acid	A	17.5	16.9	43.6
	B	7	31.1	56.2
Citric acid	A	7	17.8	58.0
	B	7	28.6	54.9
Succinic acid	A	17.5	20.4	54.1
	B	7	27.6	51.2
Malic acid	A	17.5	20.4	58.5
	B	7	26.2	49.1
Fumaric acid	A	35	16.1	50.0
	B	35	24.0	52.2
Ethanol	A	35	18.0	49.7
	B	7	26.5	52.7
Acetone	A	7	17.4	46.6
	B	35	27.5	52.5
iso-Propanol	A	35	19.9	54.5
	B	17.5	28.7	52.7
Glucose	A	7	14.1	44.5
	B	7	20.4	60.0

*1: Basal medium A....medium A_3 from which glucose was omitted.
Basal medium B....1.0% α-ketoglutaric acid and 0.5% NH_4Cl in water, pH 7.2.

*2: Each of the substrates was tested at concentrations of 35 μM/ml, 17.5 μM/ml and 7 μM/ml in each of the two kinds of basal media.
Only the representative data were demonstrated in this table.

*3: Yield of L-glutamic acid was calculated on the basis of the amounts of α-ketoglutaric acid consumed.

cells of this organism was prevented and so L-glutamic acid formed was not consumed.

DISCUSSION

The main object of this investigation was to devise a fermentative production of L-glutamic acid from α-ketoglutaric acid and an ammonium salt. Though the results, described above, may not be applied to the practical industry immediately, one may be able to conclude that it is possible to produce L-glutamic acid by fermentation. The organism, *Pseudomonas ovalis* No. 259–2, has not sufficiently powerful ability to accumulate L-glutamic acid.

It is necessary, therefore, to search further for the microorganisms which possess a marked activity in L-glutamic acid production.

The highest yield of L-glutamic acid was obtained when the cell suspension was incubated anaerobically at 30°C and the pH of the medium was maintained neutral or slightly alkaline. Under these conditions, about 60% of the original α-ketoglutaric acid were converted to L-glutamic acid and were accumulated in the medium after 24 hrs'. incubation.

It has been reported that glucose was converted 50 to 60% to α-ketoglutaric acid by fermentation[15]. Thus, about 30% of glucose may be converted to L-glutamic acid by a combined use of the two fermentative procedures, α-ketoglutaric acid producing fermentation and L-glutamic acid producing fermentation.

It should be noted here that the cells which were used in these experiments and showed a high yield of L-glutamic acid were harvested from the medium containing 1% α-ketoglutaric acid. As α-ketoglutaric acid is rather expensive material, it would be desirable for the industrial application to find out the less expensive medium which would also support the growth of cells capable of producing L-glutamic acid. However, formulation of such a media may not be so easy, for the L-glutamic acid producing activities of the cells seems to depend upon the composition of the media on which they were grown.

We have found some microorganisms capable of accumulating of L-glutamic acid in the medium which is composed of glucose, ammonium salt, minerals, and growth factors. Using these microorganisms, it seemed possible, therefore, to produce L-glutamic acid from glucose and ammonium salts by one step fermentation. The details of this observation will be reported in the future papers.

In the enzymatic procedure of L-glutamic acid production proposed by Smythe *et al*[3], the homogenate of the cells is used as the enzyme source to convert α-ketoglutaric acid to L-glutamic acid. The homogenate, however, can not be used repeatedly, because the reaction mixture is heated after incubation to stop the reaction. When the intact whole cells were used, the cells can be separated by centrifugation from the reaction mixture after the reaction was completed. Thus the repeated use of cells may be possible by merely adding the cells to the fresh reaction mixture. Though the details of the experiment were not presented in this paper, it was observed that an additional accumulation of L-glutamic acid took place when α-ketoglutaric acid and NH_4Cl were added again to the medium in which the cells of *Ps. ovalis* were grown and α-ketoglutaric acid had been almost completely consumed. This observation suggests the possibility of the repeated use of intact cells for L-glutamic acid production.

From the results of enzymological studies obtained, it would be said that L-glutamic acid is formed by *Ps. ovalis* cells through either one or both of the following pathways;

1. α-ketoglutarate+NH_3+$TPNH_2$——L-glutamic dehydrogenase⟶ L-glutamate+TPN+H_2O
2. α-ketoglutarate——TCA cycle⟶fumarate
   fumarate+NH_3——aspartase⟶L-aspartate
   L-aspartate+α-ketoglutarate——aspartic-α-ketoglutaric transaminase⟶oxalacetate+L-glutamate

However, it is quite probable that the former pathway 1 is mainly working, because the anaerobic condition was more favorable for the accumulation of L-glutamic acid.

The consumption of once accumulated L-glutamic acid by the same cells is of importance from both practical and physiological point of view. The devices to prevent this consumption are very profitable for the application of our procedure to the practical purpose.

Investigation of the mechanisms of L-glutamic acid consumption by the cells would also serve to make clear the mechanism of L-glutamic acid accumulation.

SUMMARY

1. Searches for the microorganism which are able to grow in the medium, containing α-ketoglutaric acid and ammonium chloride, and do accumulate L-glutamic acid in the medium were carried out. The accumulation of L-glutamic acid was detected in the cultures of the microorganisms belonging to the following genera;

Agrobacterium, *Aerobacter*, *Aeromonas*, *Bacillus*, *Bacterium*, *Erwinia*, *Escherichia*, *Micrococcus*, *Serratia*, *Pseudomonas*, *Xanthomonas*, *Debaryomyces*, *Hansenula*, *Mycotorula*, *Pseudosaccharomyces*, *Saccharomyces*, *Willia*, *Aspergillus*, *Penicillium* and *Rhizopus*.

A strain of *Pseudomonas ovalis* showed the highest level of L-glutamic acid accumulation. A description of the morphological and physiological properties of this strain were presented. This strain was used in the subsequent experiments.

2. A test of the effects of nitrogen sources on L-glutamic acid formation revealed that ammonium chloride was the best nitrogen source for the L-glutamic acid formation.

3. The aerobic condition was favorable for the growth of this organism, but not for the accumulation of L-glutamic acid.

4. The accumulation process of L-glutamic acid was separated from the growth phase of the organism. After the organism was grown in the medium A_3 on the shaker at 30°C for 17 to 18hrs., the cells were harvested by centrifugation and washed thrice. The washed cells were added to the fresh medium A_3, and incubated at 30°C. The initial concentration of the cells were 30 mg cells (wet weight) per ml. The highest yield of L-glutamic acid was obtained when the pH of the medium was maintained neutral or slightly alkaline and the incubation was carried out anaerobically. Within

24 to 30 hrs., about 98% of the added α-ketoglutaric acid was consumed and 30 μM L-glutamic acid per ml, an amount corresponding to 60% of the consumed α-ketoglutaric acid, were accumulated in the medium.

5. When the cell suspension was incubated aerobically or the pH of the medium was not controlled, the accumulation of L-glutamic acid remained at a lower level.

6. The enzymological studies suggested that L-glutamic acid was formed through either one, or both, of the following pathways;

1. α-ketoglutarate + $TPNH_2$ + NH_3 —— L-glutamic dehydrogenase ⟶ L-glutamate + TPN + H_2O
2. α-ketoglutarate —— TCA cycle ⟶ fumarate
   fumarate + NH_3 —— aspartase ⟶ L-aspartate
   L-aspartate + α-ketoglutarate —— aspartic-α-ketoglutaric transaminase ⟶ oxalacetate + L-glutamate

The pathway 1 seemed to be mainly working in our organism used.

7. The problems derived from this work and left in the future studies were pointed out.

ACKNOWLEDGEMENT

The authors wish to express their thanks to Assist. Prof. H. Iizuka and Mr. K. Komagata of The Institute of Applied Microbiology, The University of Tokyo, for identifying the microorganisms used in this study, to Mr. J. Kirimura of The Sericultural Experiment Station, M. A. F., for microbioassay of asparatic acid, and to The Ajinomoto Co., Inc. for supplying the chemicals.

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J. Gen Appl. Microbiol.
Vol. 3, No. 1, 1957

ENZYMIC DEGRADATION OF YEAST RIBONUCLEIC ACID AND ITS RELATED COMPOUNDS BY ASPERGILLUS ORYZAE

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Received for publication Oct ber 4, 1956

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There exist numerous reports concerning the nucleolytic enzymes, but little work has been done on the enzymes from Koji-molds. Although Iwanoff[1], Noguchi[2], and Ôtani[3] reported the degradation of thymus or yeast nucleic acid by molds or Takadiastase, they did not describe the pathways in detail. In 1955 the author first reported the results of experiments on the pathways of the enzymic degradation of ribonucleic acid by the ribonucleolytic enzyme system from *Aspergillus oryzae*. Almost simultaneously, Saruno[4] reported the results of preliminary experiments on these pathways using rice koji nucleases.

The purpose of the present paper is to show systematically the pathways in the enzymic degradation of yeast ribonucleic acid and its related compounds. It will be demonstrated that the ribonucleolytic enzyme system from *Aspergillus oryzae* contains the following enzymes: ribonucleodepolymerase acting on yeast ribonucleic acid, mononucleotide phosphatase acting on 3′-adenylic acid, 3′-guanylic acid, 3′-cytidylic acid, and 3′-uridylic acid, adenyl deaminase acting on 3′-adenylic acid and adenosine, and purine nucleoside hydrolase acting on inosine and guanosine, as well as 5 -inosinate-N-ribosidase acting specifically on the N-ribosidic linkage of 5′-inosinic acid. It is of special interest that *Aspergillus oryzae* contains thermostable ribonucleodepolymerase and a new enzyme, "5′-inosinate-N-ribosidase".

MATERIALS AND METHODS

Materials—Yeast ribonucleic acid was obtained from Kirin Research In-

stitute, and used without further purification. 5′-Inosinic acid and ribose-5-phosphate were prepared from rabbit muscle by the method of Marmur *et al*[5]. 3′-Inosinic acid was obtained by deaminating 3′-adenylic acid prepared from alkaline hydrolysis of yeast ribonucleic acid[6]. A crude preparation of ribose-3-phosphate was prepared from 3′-guanylic acid via 3′-xanthylic acid[7]. As a crude preparation of ribose-1-phosphate, the reaction mixture, which resulted from the action of the nucleoside phosphorylase fraction of rabbit liver[8] on inosine with potassium phosphate (dibasic), was used for convenience. The other nucleotides, nucleosides, purine or pyrimidine bases, sperm deoxyribonucleic acid, and ribose were obtained from commercial sources. The preparations of 3′-adenylic acid, 3′-guanylic acid, 3′-cytidylic acid, 3′-uridylic acid, 3′-inosinic acid, and ribose-3-phosphate, employed in the present study, probably contained the corresponding 2′ isomers respectively. However, these preparations were used without removal of the 2′ isomers.

Enzyme preparation—As the main source of enzymes, *Aspergillus oryzae* var. No. 13, a strain employed in soy-manufacture[9], was used. It was grown at 30°C for 10 days on a medium of the following composition: glucose, 5%; polypeptone*, 0.5%; KH_2PO_4, 0.05%; K_2HPO_4, 0.05%; $CaCl_2$, 0.04%; $MgSO_4 \cdot 7H_2O$, 0.04%. The culture filtrates or their partially purified preparations were used as the enzyme solution. The purification was carried out mainly by dialysis, salting out, and the use of organic solvents or ion exchage resins. Details on the procedure for each enzyme preparation will be described under the individual experiments. All enzyme reactions were performed in an atmosphere of air.

Paper chromatography—The following solvent systems were employed for paper chromatography (the ratios are in volume proportions): (1) n-butyl alcohol, acetic acid, and water (4:1:1), (2) saturated ammonium sulfate solution, isopropyl alcohol, and water (79:2:19), (3) n-butyl alcohol, acetic acid, and water (1:1:0.5). The chromatographic data were obtained under somewhat varying conditions but always using known standards. The technique of ascending chromatography was employed using TOYO-Roshi's No. 50 filter paper. For the separation of bases, nucleosides, and nucleotides, solvent (1) or (2) was employed at room temperature. The spots were detected by illumination of the paper with an ultraviolet lamp (SANKYO GL 15W) provided with filter-2537 Å which was made in the Laboratory of Dr. Iwase, the Scientific Research Institute, Ltd.** The spots detected were eluted overnight in 5.0 ml of 0.1N hydrochloric acid at 35°C, and the amount present was determined by ultraviolet absorption measurements using a Hitachi model EPU 2 spectrophotometer.

Chromatographic separation of ribose phosphates or ribose was carried

* Besides polypeptone, asparagine was also effective as a nitrogen source.

** I wish to express my thanks to Dr. Iwase for kindly supplying the ultraviolet filter-2537Å for this study.

out with solvent (3) at a temperature below 0°C. The technique used was practically identical with that described by Tarr[10]. The chromatograms were sprayed with aniline hydrogen phthalate reagent containing 0.5 ml concentrated hydrochloric acid/100 ml, and heated.

Paper electrophoresis—Paper electrophoresis was mostly performed at room temperature in 10% acetic acid either (A) with 300V and about 0.2 mA/cm for 5.5 hours, the starting line being 10 cm from the end of the anode side, or (B) with 400V and about 0.3 mA/cm for 5.0 hours, the starting line being 5 cm from the end of cathode side. Conditions (A) and (B) were employed in the experiments of RESULTS (2) and (4), respectively. The distance from one end to the other was 31 cm. The direction to the anode side from the starting line was designated as plus. Caffeine was employed as a standard of reference in order to determine the velocity of electroosmotic flow. The spots were detected by the same method as used in chromatography. Caffeine was detected under an ultraviolet lamp. A combination of paper chromatography and electrophoresis was shown to be effective for identification of the hydrolytic products of ribonucleic acid. (See Figures 7 and 11 for examples.)

Analytical methods—Inorganic and organic phosphates were determined according to the Fiske-Subbarow method[11]. Optical density was read at 750 mμ. In order to determine inorganic phosphate in the presence of acid-labile phosphate, the Lowry-Lopez method[12] was employed (Table 8). The colorimetric method of Nelson[13] to observe the appearance of free ribose or ribose phosphate was employed, except that the treatments with barium hydroxide and zinc sulfate were omitted. Under these conditions any significant difference in color yield (optical density at 520 mμ) between ribose and ribose-5-phosphate was not recognized. The enzymic activities were measured as indicated under the individual experiments.

Table 1. Time-course of enzymic dephosphorylation of ribo- or deoxyribonucleic acid.

	Incubation time (hr) / Substrate	0	3.5	7.0
Inorg. P liberated (μg/ml)	RNA	0 (0%)*	60.0 (25.4%)*	125.5 (53.2%)*
	DNA	0 (0%)**	19.5 (8.2%)**	23.2 (9.8%)**
	RNA+DNA	0	41.7	91.3

Composition of reaction mixture: 1 ml of substrate solution (RNA or DNA, 17 mg), 2 ml of enzyme solution (culture filtrates of *Aspergillus oryzae A*), and M/35 veronal buffer (pH 4.0). Total volume 6 ml. Incubation temp.: 30°C.

* (Inorg. P liberated)×100/RNA-P added.

** (Inorg. P liberated)×100/DNA-P added.

RESULTS

(1) *Mechanism of enzymic liberation of inorganic phosphate from yeast ribonucleic acid by Aspergillus oryzae.*

Aspergillus oryzae A cleaved yeast ribonucleic acid more strongly than *Bacillus natto, Escherichia coli,* or *Staphylococcus candidus.* Culture filtrates and mycelial extracts caused the disappearance of ribonucleic acid, liberated inorganic phosphate and small molecular compounds which absorbed ultraviolet light, and increased the acidity of the reaction mixture. Sperm deoxyribonucleic acid was oberved to be only very slightly degraded by *Aspergillus oryzae A.* Moreover it is suggested from Table 1† that deoxyribonucleic acid inhibits the degradation of ribonucleic acid by the *Aspergillus oryzae* enzyme system in an early stage. The investigation of this possibility will be the subject of future research.

The results obtained with *Aspergillus oryzae* var. No. 13, as well as with Takadiastase, were also essentially similar to those with *Aspergillus*

Table 2. Influence of ion exchange resin (Amberlite) on ribonucleolytic activities.

	Condition of resin	Resin used	IR-120	IR-112	IRC-50	IRA-400	IR-4B
U.R. sol. P(μg) liberated from RNA (3mg) by resin-treated enzyme (0.2ml)	(1) free	Inorg. P	82	0	0	60	0	0
		Total P	211	$\leqq$120	21	199	0	$\geqq$157
	(2) buffered (pH 4.0)	Inorg. P	82	65	75	64	65	53
		Total P	211	210	207	199	200	171
	(3) buffered (pH 8.4)	Inorg. P	82	68	68	64	47	0
		Total P	211	200	188	215	180	175

Composition of reaction mixture: 4 ml of substrate solution (15 mg of RNA in N/10 acetate buffer, pH 4.0) and 1 ml of enzyme soln.* after contact with each resin at 10°C for 17 hr. Total volume 5 ml. pH 4.0. Incubation time: 15 min., temp.: 60°C.

* contains 0.5 ml of concentrated culture filtrate.

oryzae A. It is demonstrated from the following experiments with *Aspergillus oryzae* var. No. 13 that the results observed above are caused by the action of two enzyme systems, ribonucleodepolymerase and ribonucleophos-

† The following abbreviations are used throughout all figures and tables: yeast ribonucleic acid, RNA; sperm deoxyribonucleic acid, DNA; ribonucleodepolymerase system, RN-depolymerase; ribonucleophosphatase system, RN-phosphatase; uranyl reagent, U.R.; inorganic orthophosphate, inorg. P; inorganic pyrophosphate, PP; organic phosphate, org. P; 3′-(yeast) adenylic acid, A3P; 5′-(muscle) adenylic acid, A5P; 3′-guanylic acid, G3P; 3′-inosinic acid, I3P; 5′-inosinic acid, I5P; 3′-cytidylic acid, C3P; 3′-uridylic acid, U3P; adedosine, AR; guanosine, GR; inosine, HxR; cytidine, CR; uridine, UR; adenine, A; guanine, G; hypoxanthine, Hx; xanthine, X; cytosine, C; uracil, U; thymine, T; ribose-1-phsphate, R1P; ribose-3-phosphate, R3P; ribose-5-phosphate, R5P.

phatase.

(a) *Properties of the Aspergillus ribonucleodepolymerase and ribonucleophosphatase.*

In order to establish the mechanism of enzymic liberation of inorganic phosphate from yeast ribonucleic acid by *Aspergillus oryzae* var. No. 13, several experiments were carried out. The results are shown in Tables 2

Table 3. Influence of inhibitors on ribonucleolytic activities.

Additions (final concn. 10^{-3}M)	U.R. sol. P. liberated from RNA (%)	
	Inorg P	Total P (RN-depolymerase)
—	100*	100*
Na_2HAsO_4	100	100
$NaAsO_2$	100	100
NaF	0	87.6
KCN	96.5	94.4
CH_2ICOOH	104.7	94.8
Ninhydrin	87.2	90.8
$CuSO_4$	29.8	46.4
$ZnSO_4$	76.7	74.6
$CaCl_2$	100	104
NaCl	100	100
$MnSO_4$	100	100
$HgCl_2$	20	20
$FeCl_3$	41	49.3
$MgSO_4$	100	101
$PbCl_2$	28	39.4
$AlCl_3$	54	61
$CdCO_3$	100	108

* Without additions 248 μg of U.R. sol. total P (containing 57 μg of inorg. P) was liberated from 5 mg. of RNA during incubation. (Incubation temp.: 60°C, time: 30 min., pH: 4.0).

and 3, and in Figures 1 through 3. These findings support the view that the *Aspergillus oryzae* enzymes liberating inorganic phosphate from ribonucleic acid consist of two systems: ribonucleodepolymerase, thermostable, which degrades ribonucleic acid into intermediates soluble in uranyl reagent (0.25% uranyl acetate in 2.5% trichloroacetic acid solution)† and ribonucleophosphatase, thermolabile, which degrades the intermediates into nucleosides and inorganic phosphate.

† This reagent was prepared by a slight modification of MacFadyen's reagent[14].

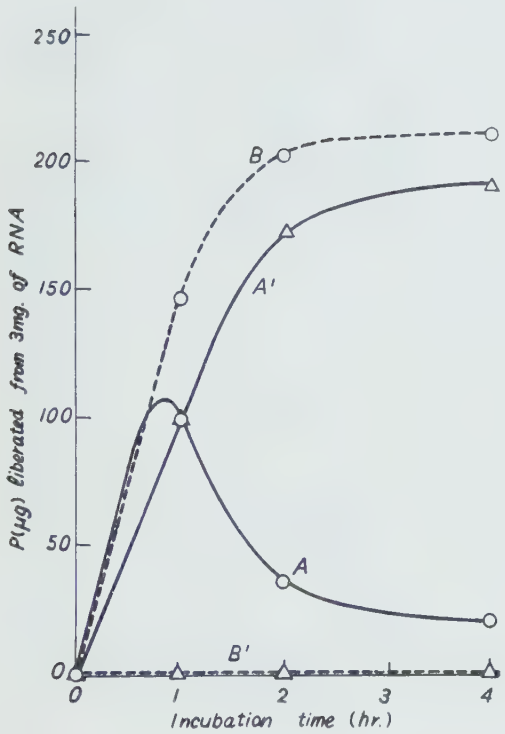

Fig. 1. Influence of dialysis on ribonucleolytic activities.

A : U.R. sol. org. P formed by undialyzed enzyme

A′: Inorg. P formed by undialyzed enzyme

B : U.R. sol. org. P formed by dialyzed enzyme

B′: Inorg. P formed by dialyzed enzyme

As the enzyme solution, culture filtrate (curves A and A′) or its non-dialyzable fraction* (curves B and B′) was used, and was incubated with an equal volume of substrate solution (RNA 6 mg/1 ml of N/10 acetate buffer, pH 4.0) at 45°C. After 1, 2, and 4 hr, 2 ml of each reaction mixture was mixed with 2 ml of U.R.. Org. P (curves A and B) and inorg. P (curves A′ and B′) of the filtrate were determined.

* The culture filtrate was dialyzed against running water over-night.

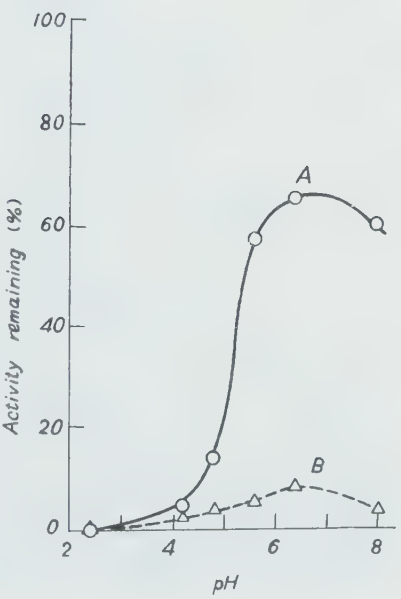

Fig. 2. Influence of Japanese acid clay at various pH values on ribonucleolytic activities.

Curve A: Activity of U.R. sol. P formation from RNA. (RN-depolymerase)

Curve B: Activity of inorg. P liberation from RNA. (RN-phosphatase)

Concentrated culture filtrate was mixed with Japanese acid clay (0.2 g/ml) at 0°C for 30 min. The activities of the filtrate were measured by incubation with RNA (temp.: 60°C, time: 30 min., pH: 4.0).

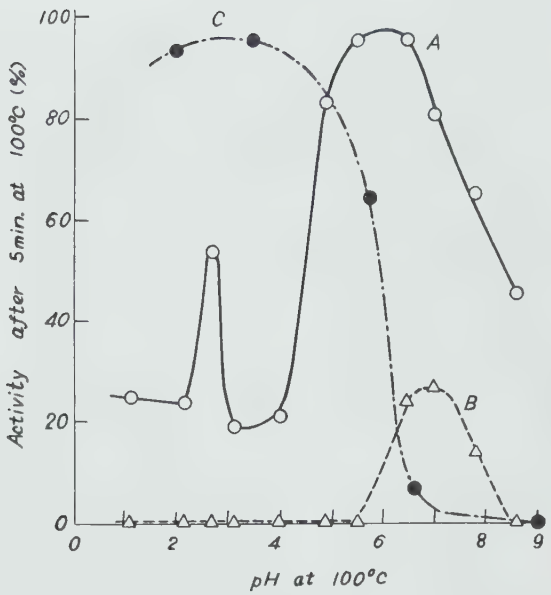

Fig. 3. Effect of pH on stability of ribonucleolytic enzymes at 100°C.

Curve A: Activity of U.R. sol. P formation from RNA. (RN-depolymerase)

Curve B: Activity of inorg. P liberation from RNA. (RN-phosphatase)

Incubation temp.: 60°C, time: 30 min., pH: 4.0.

Culture filtrate was used as enzyme solution.

Curve C: Activity of crystalline ribonuclease (pancreas) according to Kunitz[16].

$$\text{RNA} \xrightarrow[\text{Ribonucleodepolymerase (thermostable)}]{} \underset{\text{intermediates}}{\text{Uranyl reagent soluble}} \xrightarrow[\text{Ribonucleophosphatase (thermolabile)}]{} \text{Nucleosides+Inorg. P} \quad (1)$$

(The names ribonucleodepolymerase and ribonucleophosphatase were employed according to the terminology proposed by Laskowski.[15])

The activity of ribonucleodepolymerase was still preserved after dialysis and contact with a weak base anion-exchange resin (Amberlite IR-4B) or Japanese acid clay, while ribonucleophosphatase was inactivated by these treatments, as shown in Figures 1 and 2, and Table 2. Furthermore, it is clear from Table 3 that sodium fluoride strongly inhibits ribonucleophosphatase activity but inhibits ribonucleodepolymerase hardly at all. Arsenate,

arsenite, cyanide, monoiodoacetate and ninhydrin did not markedly inhibit the *Aspergillus* ribonucleolytic enzyme system liberating inorganic phosphate from ribonucleic acid.

(b) *Influence of temperature and pH on the action of partially purified Aspergillus ribonucleodepolymerase preparation.*

As was shown by the results given in Figure 3, the *Aspergillus oryzae* ribonucleodepolymerase has a remarkable thermostability which is especially notable from pH 5.5 to pH 6.5. The experimental results with a partially purified ribonucleodepolymerase preparation were essentially similar to those with a crude preparation in the experiment given in Figure 3. The partially purified ribonucleodepolymerase preparation employed in the above experiment was obtained as follows: To the concentrated culture filtrates of *Aspergillus oryzae* var. No. 13 four volumes of ethanol were added. The resulting precipitate was extracted with cold water. The extracts were subjected to salting out with fully saturated ammonium sulfate. Most of the ribonucleodepolymarase activity was left in the solution. (When salting out was carried out after dialysis of a more concentrated solution, the *Aspergillus* ribonucleodepolymerase was largely precipitated with fully saturated ammonium sulfate. (See "(2) (b)" or "(3)")). The solution was dialyzed against distilled water, concentrated again *in vacuo*, then precipitated by the addition of four volumes of ethanol, and at last dissolved in distilled water. The resulting clear and colorless solution was employed as the partially purified ribonucleodepolymerase preparation. This preparation did not con-

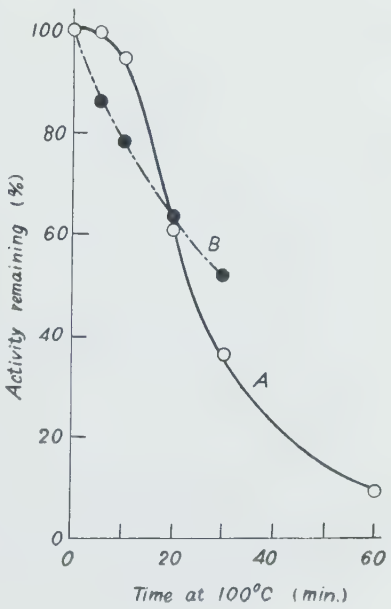

Fig. 4. Inactivation of RN-depolymerase at 100°C in N/20 sodium acetate solution.
(Partially purified RN-depolymerase preparation was use.)
Curve A: Activity of RN-depolymerase. (*Asp. oryzae*)
Curve B: Activity of crystalline ribonuclease (pancreas) according to Kunitz. [Heat treatment was carried out in 0.02M acetate buffer, pH 5.8.][16]

tain to any appreciable extent deoxyribonucleolytic enzymes or ribonucleophosphatase. Figure 4 shows the effect of heating time on the ribonucleodepolymerase activity of this preparation. In 0.05N sodium acetate solution, 94% of the activity was retained after heating for 10 minutes at 100°C. The thermostability of *Aspergillus* ribonucleodepolymerase is compared with that of pancreas ribonuclease[16] in Figures 3 and 4.

The optimum conditions for the activity of ribonucleodepolymerase were found to be at about 60°C and pH 4.0. The results with the partially purified preparation are given in Figures 5 and 6.

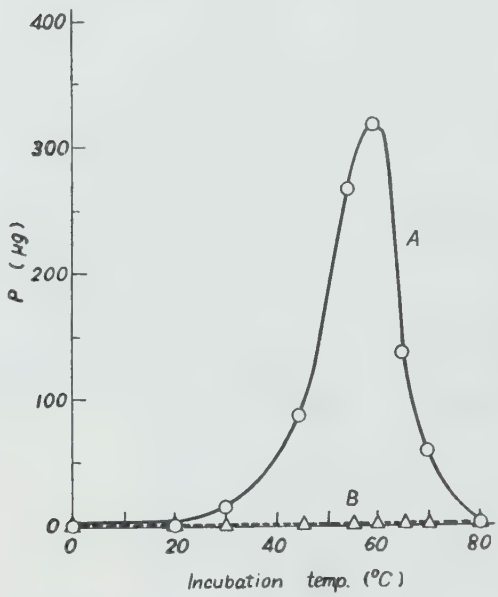

Fig. 5. Activity of RN-depolymerase at various temperatures.

(Partially purified RN-depolymerase preparation was used.)

Incubation time: 30 min., pH: 4.0.

Curve A: U.R. sol. org. P formed from 6 mg of RNA by 0.2 ml of the enzyme preparation.

Curve B: Inorg. P liberated from RNA.

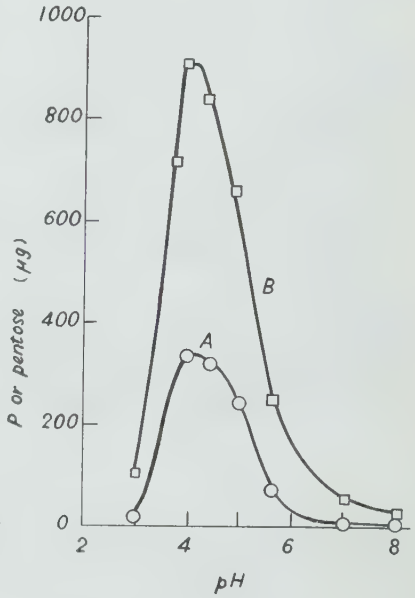

Fig. 6. Activity of RN-depolymerase at various pH values.

(Partially purified RN-depolymerase preparation was used.)

Incubation temp.: 60°C, time: 30min.

Curve A: U.R. sol. org. P formed from 6 mg of RNA by 0.2 ml of the enzyme preparation.

Curve B: U.R. sol. and orcinol-reactive pentose formed from RNA.

Thus the existence of a thermostable ribonucleodepolymerase, capable of transforming yeast ribonucleic acid to products soluble in uranyl reagent without the liberation of inorganic phosphate, was demonstrated for the first time in *Aspergillus oryzae*. The suggested mechanism of the action of this enzyme (or a system of enzymes) will be given in a later section.

(c) *A simple method for the simultaneous detection of ribonucleolytic*

enzymes splitting phosphate bonds.

For detection of ribonucleodepolymerase and ribonucleophosphatase in many samples, a simple procedure requiring only a small amount of sample was devised. The procedure consists of the following steps:

1. 3% agar solution containing ribonucleic acid (1 mg/ml) and N/10 acetate buffer, pH 4.0, is poured into a petri dish, and solidified, the thickness being 4 mm.

2. Filter paper disks, 6.5 mm in diameter, are prepared, and each of them is soaked in the sample solution to be tested. (When the enzyme is separated on filter paper, for example by electrophoresis, appropriate sections cut out are used as such.)

3. Excess solution is removed by a dry filter paper, and the disks are placed on the agar plate.

4. The plate containing disks is then incubated at 45°C for an appropriate length of time, during which the following changes may occur:

i. If the disk lacks both ribonucleodepolymerase and phosphatase, the area under the disk undergoes no change.

ii. If the disk contains only ribonucleodepolymerase but not phosphatase, the area under the disk becomes soluble in uranyl reagent, without, however, liberating inorganic phosphate.

iii. If the disk contains both ribonucleodepolymerase and phosphatase, the area under the disk becomes soluble in uranyl reagent, and at the same time inorganic phosphate is formed.

5. After the incubation, the disks are removed, and uranyl reagent is added to the agar plate in order to detect depolymerase. In case (i), the whole agar plate will become turbid, while in case (ii) and (iii), the area under the disk will remain clear. According to the degree of clarity the relative activity of ribonucleodepolymerase can be gauged.

6. Uranyl reagent is then removed, and molybdate I[11] is added to the agar plate in order to detect phosphatase. After standing for 5 minutes, molybdate I is removed and a solution of aminonaphthol sulfonic acid is added. In cases (i) and (ii), no change will occur, while in case (iii), a blue color caused by inorganic phosphate will develop in the clear area under the disk.

This method proved convenient and satisfactorily reliable in preliminary determination of the enzymic activities in various fractions during the course of purification. At least 10 samples could be readily and simultaneously tested using a petri dish 8 cm in diameter.

(2) *Identification of enzymic degradation products of yeast ribonucleic acid, nucleotides, nucleosides, and purine or pyrimidine bases.*

To get further insight into the course of events involved in the reaction shown by Eq. (1), attempts were made to clarify the enzymic degradation products of yeast ribonucleic acid and its related compounds.

(a) *Identification of degradation products formed by the action of ribo-*

nucleodepolymerase upon yeast ribonucleic acid.

As described above, *Aspergillus* ribonucleodepolymerase is much more stable than ribonucleophosphatase. Therefore, it is not difficult to obtain a preparation of depolymerase free from ribonucleophosphatase. (The experiments with such a preparation have already been shown in Figures 4 through 6). In this section a preparation obtained by the following procedure was used: Culture filtrates of *Aspergillus oryzae* var. No. 13 were concentrated *in vacuo*, and then dialyzed against distilled water. The resulting solution was passed through an Amberlite IRA-400 (100 mesh) column adjusted to pH 7.0. Ribonucleophosphatase activity was almost completely removed by this procedure. (Contact with the same resin in a beaker was less effective as described in Table 2.) On the other hand, only a very little difference in the activity of ribonucleodepolymerase was noticed before and after resin treatment. The resulting solution was concentrated *in vacuo*.

About 0.5 ml of this preparation was incubated with 110 mg of yeast

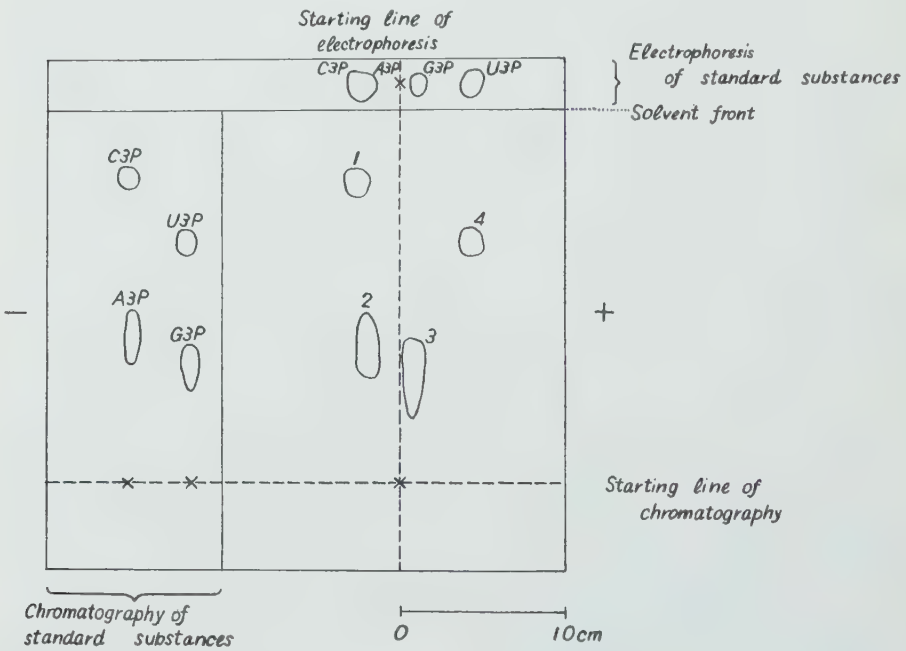

Fig. 7. Separation of the products formed by the action of RN-depolymerase on RNA.

The mixture of the degradation products of RNA was subjected to electrophoresis on paper, and the dried paper was developed in ascending fashion with solvent (2) in a direction perpendicular to that of the electrophoresis.

Four spots corresponding to C3P (1), A3P (2), G3P (3), and U3P (4) were recognized.

ribonucleic acid in N/10 acetate buffer, at pH 4.0 and 60°C, the total volume being 11 ml. Organic phosphate soluble in uranyl reagent was found to increase linearly for 30 minutes, then slowly. The amount of organic phosphate liberated in 1 and 26 hours was 70 and 76%, respectively, of the total phosphate. (This reaction mixture was soluble in 1.25% trichloroacetic acid, and contained a trace of inorganic phosphate.) Five ml of the reaction mixture was then concentrated *in vacuo*, and the precipitate formed was removed.

The compounds absorbing ultraviolet light in the region of 260 mμ were separated on filter paper. As shown in Figure 7, the degradation products contained four substances absorbing ultraviolet light. The electrophoretic mobilities and R_F values in solvent (2) of these substances were similar to those of 3′-cytidylic acid, 3′-adenylic acid, 3′-guanylic acid and 3′-uridylic acid. The R_F values in solvent (1) of these substances were approximately 0, again coinciding with the values shown by the four mononucleotides. As shown in Figure 8, the ultraviolet absorption curve of each spot was essentially similar to that of the corresponding spot of mononucleotide.

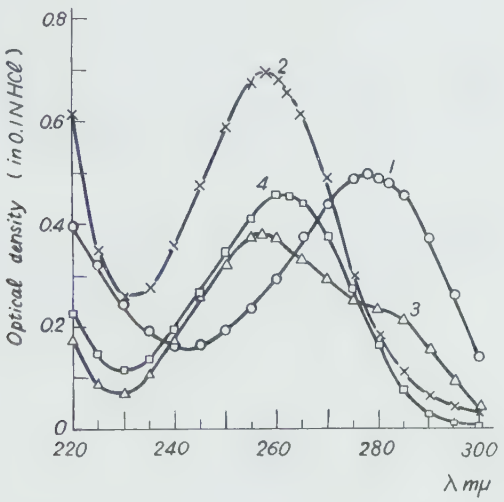

Fig. 8. Ultraviolet absorption spectra of the products formed by the action of RN-depolymerase on RNA.

Curves 1, 2, 3, and 4 are the absorption spectra of the solutions extracted from spots 1, 2, 3, and 4 (shown in Fig. 7), and are identical with the spectra of C3P (λ max 278 mμ), A3P (λ max 257 mμ), G3P (λ max 257 mμ), and U3P (λ max 262 mμ), respectively.

These results indicate that the phosphate compounds soluble in uranyl reagent are composed of adenylic acid, guanylic acid, cytidylic acid and uridylic acid, and therefore that most of the yeast ribonucleic acid is degraded to these four mononucleotides by the ribonucleodepolymerase investigated.

The molar ratio was calculated, according to the values given by Dorough and Seaton[17], to be as follows: cytidylic acid: adenylic acid: guanylic acid: uridylic acid=8.5:10.0:6.8:10.1. (In addition to the compounds corresponding to these mononucleotides, there appeared only minute amounts of compounds corresponding to adenosine, guanosine, cytidine and uridine. These nucleosides might have been formed by a trace of phosphatase in the enzyme preparation.) The mononucleotide products, which were formed by the action of the heat-treated enzyme preparation (100°C for 30 minutes) upon ribonucleic acid, were also found to be adenylic, guanylic, cytidylic, and uridylic acids.

To make clear the position of the ester linkage in the mononucleotides formed by the action of ribonucleodepolymerase upon ribonucleic acid, the following experiments were performed. The degradation products, four mononucleotides, were separated by paper electrophoresis using 0.02M citrate buffer of pH 3.5[18]. Separated four spots corresponding to the four mononucleotides were cut out, and placed on the starting line of another piece of filter paper. A second paper electrophoresis was carried out using M/10 borate buffer of pH 9.2,[19] with 150 V and 0.6 mA/cm at room temperature for 8 hours. The results are presented in Table 4. The migration of each mononucleotide was essentially similar to that of a 3′-nucleotide. Generally a 5′-nucleotide is thought to form a complex with borate and accordingly migrates more rapidly than a corresponding 3′-nucleotide. This was actually the case in this experiment, in which 5′-adenylic acid migrated more

Table 4. Paper electrophoresis in borate buffer of four mononucleotides resulting from the action of RN-depolymerase on RNA.

	Distance migrated from origin to anode side (cm)		
	Standard		Degradation product
	3′-	5′-	
Adenylic acid	+11.8	+13.5	+11.9
Guanylic acid	+13.6		+13.6
Cytidylic acid	+12.0		+12.0
Uridylic acid	+15.2		+15.0

Starting line was at 5 cm from the end on the cathode side, and 26 cm from the end on the anode side.

rapidly than 3′-adenylic acid. It seems possible, therefore, that the four mononucleotides resulting from the action of *Aspergillus* ribonucleodepolymerase on yeast ribonucleic acid are not 5′-nucleotides, but are 3′- or 2′-nucleotides. Further support to this assumption is given by the following observation. The electrophoretic mobilities of both 3′-mononucleotides and the mononucleotides formed by the enzymic action were not modified by treatment with M/10 sodium periodate. Consumption of periodate by these

nucleotides was not observed spectrophotometrically. On the contrary, the electrophoretic results indicated that 5′-adenylic acid was decomposed by sodium periodate to a positive compound absorbing ultraviolet light. Remarkable consumption of periodate by 5′-adenylic acid was observed. The details of these results will be reported elsewhere.

(b) *Identification of degradation products formed by the action of the ribonucleolytic enzyme system upon yeast ribonucleic acid and its related compounds.*

In this series of experiments, an enzyme preparation containing the entire ribonucleolytic enzyme system was used. The procedure of preparing the enzyme sample was as follows: culture filtrates of *Aspergillus oryzae* var. No. 13 were concentrated *in vacuo*. The material was kept at a temperature below 0°C for a few days, and the resulting precipitate was removed. To the clear solution four volumes of ethanol were added. The precipitate was dissolved in distilled water, dialyzed moderately against distilled water, concentrated *in vacuo*, and then subjected to salting out with saturated ammonium sulfate. The precipitate was dissolved in distilled water, and

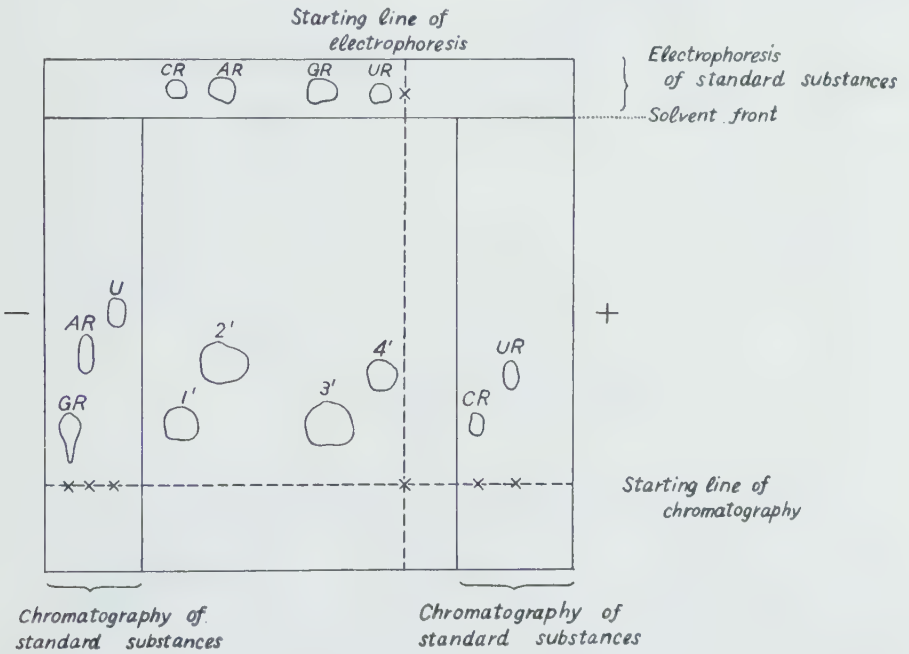

Fig. 9. Separation of the products formed by the action of the ribonucleolytic enzyme system on RNA.

The degradation products of RNA were separated on paper according to the procedure described in Fig. 7. Chromatographic solvent (1) was employed.

Four spots corresponding to CR (1′), AR (2′), GR (3′), and UR (4′) were recognized.

employed as the enzyme preparation.

1. *The action upon yeast ribonucleic acid.*

Two ml of the enzyme preparation was incubated at 60°C with 50 mg of yeast ribonucleic acid in N/10 acetate buffer of pH 4.0. (Total volume: 10 ml.) Within 8 hours, all phosphate in the reaction mixture was transformed into inorganic phosphate. Then 6 ml of the reaction mixture was concentrated to 1 ml *in vacuo*, and subjected to paper electrophoresis, chromatography, and spectrophotometry as described before.

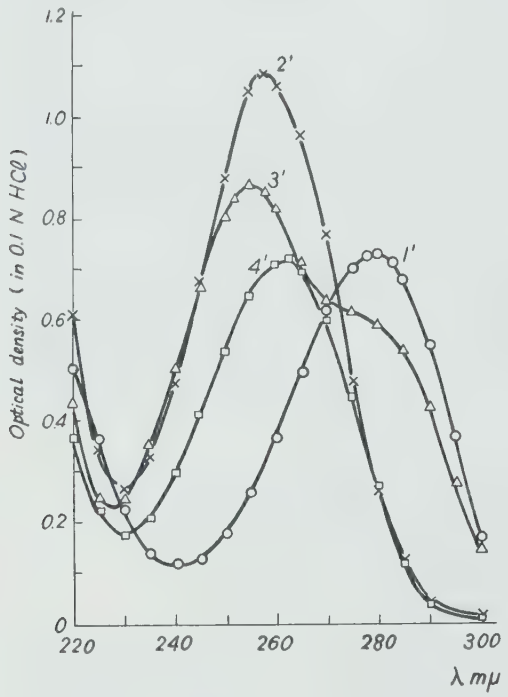

Fig. 10. Ultraviolet absorption spectra of the products formed by the action of the ribonucleolytic enzyme system on RNA.

Curves 1′, 2′, 3′, and 4′ are the absorption spectra of the solutions extracted from spots 1′, 2′, 3′, and 4′ (shown in Fig. 9), and are identical with the spectra of CR (λ max 280 mμ), AR (λ max 258 mμ), GR (λ max 256 mμ), and UR (λ max 262 mμ), respectively.

The degradation products contained four compounds absorbing ultraviolet light. These compounds corresponded to cytidine, adenosine, guanosine, and uridine, respectively. The results are shown in Figures 9 and 10. The R_F values in solvent (2) for these substances were 0.68, 0.25, 0.29, and 0.57, values which are similar to those of cytidine, adenosine, guanosine, and uridine, respectively. These results indicate that yeast ribonucleic acid is degraded by the *Aspergillus* ribonucleolytic enzyme system to four nucleosides and inorganic phosphate via four mononucleotides. Therefore ribonucleophosphatase is probably a phosphatase acting on mononucleotides. The molar ratio was calculated to be as follows: cytidine: adenosine: guanosine: uridine=7.3:10.0:9.3:9.5.

During the incubation only the action of ribonucleodepolymerase and

mononucleotide phosphatase was recognized. To clarify further the degradation pathways of mononucleotides, the respective mononucleotides, nucleosides, and bases were incubated with the enzyme preparation. The results obtained are given in the next section.

2. *The action upon nucleotides and nucleosides.*

A solution of 3 mg of each substrate was incubated with 0.1 ml† of the enzyme preparation in N/10 acetate buffer of pH 4.0 at 45°C for 6 hours or more. The total volume was 0.5 ml. The nature of the resulting products, which absorbed ultraviolet light, was investigated by the procedures previously described.

i. *Enzymic degradation of 3′-cytidylic acid*

The electrophoretic mobility, R_F values and ultraviolet absorption curve

Table 5. Identification of the products formed by the action of the ribonucleolytic enzyme system on ribonucleotides and nucleosides.

	Compd.*	Distance migrated from origin by electrophoresis (cm)	R_F Solvent (1)	R_F Solvent (2)	λ max in 0.1N HCl (mμ)	Corresponding compd.
	(C3P)′	−13.5	0.17	0.68	280	CR
	(CR)′	−13.5	0.17	0.68	280	CR
(i)	C3P	− 3.3	0.03	0.83	278	
	CR	−13.5	0.18	0.68	280	
	C	−19.4	0.29	0.65	275	
	(U3P)′	− 2.0	0.31	0.57	262	UR
	U3P	+ 4.0	0.03	0.64	262	
(ii)	Us	− 2.0	0.31	0.57	262	
	U	− 2.0	0.47	0.46	260	
		−13.0	0.23	0.25	249	G
	(G3P)′	− 5.4	0.16	0.27	255	GR
		+ 1.0	0.01	0.31	258	G3P
		−13.0	0.25	0.24	250	G
(iv)	(GR)′	− 5.5	0.16	0.27	256	GR
	G3P	+ 0.8	0.01	0.32	257	
	GR	− 5.5	0.16	0.28	256	
	G	−13.0	0.25	0.25	249	

* (C3P)′, (CR)′, (U3P)′, (G3P)′, and (GR)′ are symbols for the products formed by the action of ribonucleolytic enzyme system on 3′-cytidylic acid, cytidine, 3′-uridylic acid, 3′-guanylic acid, and guanosine, respectively.

† When adenylic or guanylic acid was employed as substrate, 0.05 ml of the enzyme preparation was used.

of the 3′-cytidylic acid degradation product were remarkably similar to those of cytidine and differed from those of cytosine. Cytidine did not change its properties upon incubation with the ribonucleolytic enzyme system. These results, given in Table 5 (i), indicate that 3′-cytidylic acid is decomposed by the enzyme system of *Aspergillus oryzae* to cytidine but further decomposition does not occur.

$$\text{3′-Cytidylic acid} \longrightarrow \text{Cytidine} \qquad (2)$$

ii. *Enzymic degradation of 3′-uridylic acid*

The degradation product of 3′-uridylic acid was identified as uridine. It was distinguished clearly from uracil by the R_F values. These results, given in Table 5 (ii), indicate that 3′-uridylic acid is decomposed by the enzyme system to uridine, but no further degradation occurs.

$$\text{3′-Uridylic acid} \longrightarrow \text{Uridine} \qquad (3)$$

iii. *Enzymic degradation of 3′-adenylic acid*

The partial degradation products of 3′-adenylic acid (reaction time, 1 hr)

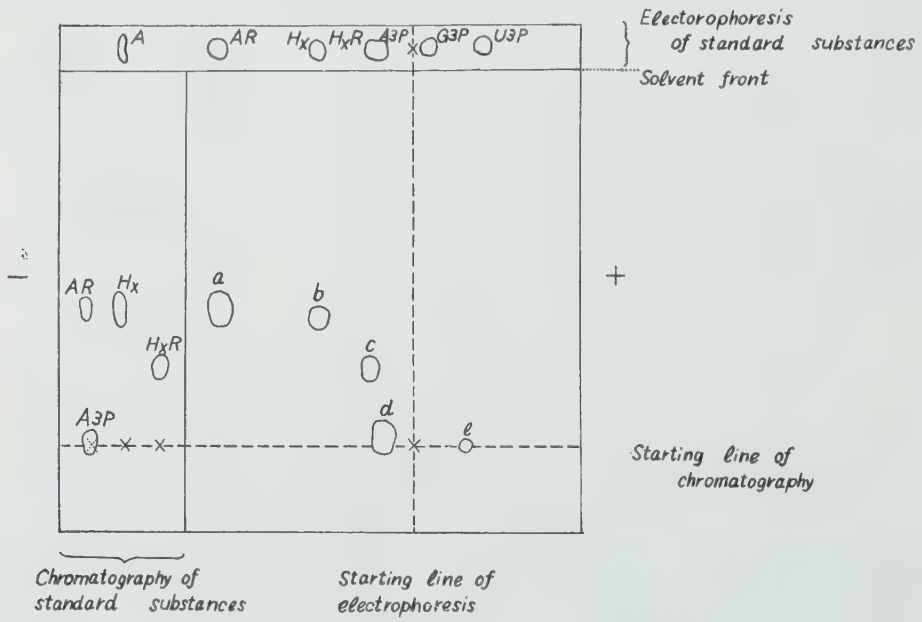

Fig. 11. Separation of the products formed by the action of the ribonucleolytic enzyme system on 3′-adenylic acid.

The products resulting from partial degradation of A3P were separated on paper according to the procedure described in Fig. 7. Chromatographic solvent (1) was employed.

Five spots corresponding to AR (a), Hx (b), HxR (c), A3P (d), and I3P (e) were recognized.

were separated on paper as shown in Figure 11. Five spots corresponding to adenylic acid, adenosine, inosine, hypoxanthine, and inosinic acid were found. The electrophoretic mobility of the compound corresponding to spot *e* was ascertained to be essentially similar to that of inosinic acid. A spot corresponding to adenine was not recognized. The absorption maxima for spots a, b, c, d and e were observed at 258 mμ, 250 mμ, 249 mμ, 257 mμ, and 249 mμ respectively. These values correspond to those of adenosine, hypoxanthine, inosine, 3′-adenylic acid, and inosinic acid, respectively. The R_F values in solvent (2) for these substances were 0.22 (a), 0.32 (b), 0.46 (c), 0.42 (d), and 0.57 (e), values which were similar to those of adenosine, hypoxanthine, inosine, 3′-adenylic acid and inosinic acid, respectively.

The degradation products of adenosine were identified as inosine and hypoxanthine, by the procedures described above. The compound corresponding to hypoxanthine was also obtained as the degradation product of inosine.

The time-course of degradation of 3′-adenylic acid was investigated as follows: 3′-adenylic acid and the enzyme preparation were incubated with and without sodium fluoride, M/400 in final concentration. At various incubation times, 0, 10, 33, and 420 minutes, 0.1 ml aliquots were removed, and separated on paper into components absorbing ultraviolet light. Without sodium fluoride the degradation of 3′-adenylic acid was observed to be accompanied by an increase in adenosine, inosine, and hypoxanthine in the early stage. While adenosine and inosine began to decrease after 33 minutes of incubation, hypoxanthine continued to increase during the whole incubation time. In the first 10 minutes about 7% of the adenylic acid was found to be converted to inosinic acid. After that, the inosinic acid did not increase further, but decreased gradually. On the other hand, when the incubation was carried out in the presence of sodium fluoride, the decrease of adenylic acid, and the increase of adenosine, inosine and hypoxanthine proceeded more slowly, but inosinic acid accumulated with time. At the end of 420 minutes' incubation, the ratio of adenylic acid, hypoxanthine, and inosinic acid was 0:81.3:1.9 in the absence of sodium fluoride and 26.2:41.0:28.6 in the presence of sodium fluoride. Adenine did not appear during incubation with or without sodium fluoride.

This series of experiments shows clearly that the degradation of 3′-adenylic acid by *Aspergillus oryzae* var. No. 13 proceeds in the following manner:

$$\text{3′-Adenylic acid} \begin{cases} \nearrow \text{Adenosine} \\ \searrow \text{3′-Inosinic acid} \end{cases} \begin{cases} \searrow \\ \nearrow \end{cases} \text{Inosine} \rightarrow \text{Hypoxanthine} \qquad (4)$$

It seems likely that in the absence of sodium fluoride, 3′-adenylic acid is degraded mainly via adenosine, and that 3′-inosinic acid, which is formed only slightly, will be subjected to further degradation. (It is demonstrated in section, "(4)" that 3′-inosinic acid is converted to inosine and inorganic phosphate.) In the presence of sodium fluoride, the action of phosphatase

on adenylic and inosinic acids is inhibited. Thus inosinic acid formed by the action of adenylic acid deaminase might accumulate.

iv. *Enzymic degradation of 3′-guanylic acid*

The degradation products of 3′-guanylic acid were identified as guanosine and guanine, and the degradation product of guanosine was identified as guanine. The results are shown in Table 5 (iv). Figure 12 shows the time-course of the degradation of guanylic acid, along with the formation of guanosine and guanine. Under the present experimental conditions addition of sodium fluoride did not cause the formation of xanthylic acid. Deamination of guanosine or guanine was not also observed.

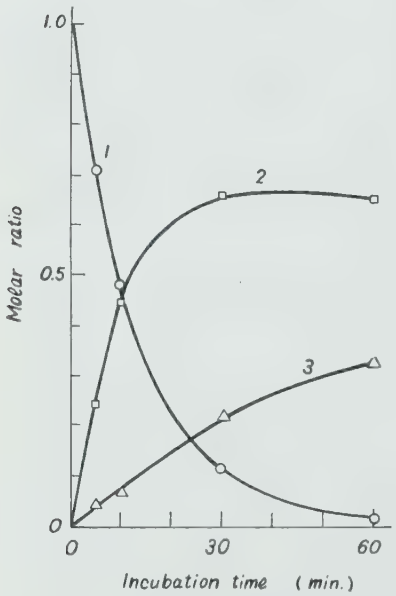

Fig. 12. Enzymic degradation of 3′-guanylic acid by the ribonucleolytic enzyme system.

1, ——○——, G3P/(G3P+GR+G).
2, ——□——, GR/(G3P+GR+G).
3, ——△——, G/(G3P+GR+G).

Composition of reaction mixture: 0.45 ml of substrate soln. (3 mg of guanylic acid in N/10 acetate buffer, pH 4.0) and 0.05 ml of enzyme soln. Total volume 5 ml.

Incubation temp.: 45°C.

These findings suggest that the degradation of 3′-guanylic acid is carried out as follows:

$$\text{3′-Guanylic acid} \longrightarrow \text{Guanosine} \longrightarrow \text{Guanine} \qquad (5)$$

(c) *Change in ultraviolet absorption spectra of various nucleotides, nucleosides and purine or pyrimidine bases during incubation with the ribonucleolytic enzyme system.*

Solutions* of various nucleotides, nucleosides and purine or pyrimidine bases were incubated with the ribonucleolytic enzyme system**. Table 6 summarizes

* Their concentrations were adjusted to show absorbance maxima at approximately 0.5.

** The preparation used in the foregoing experiment ("(2) (b)") was also employed in this experiment.

Table 6. Change of ultraviolet absorption spectra of RNA derivatives by the ribonucleolytic enzyme system in N/20 acetate buffer, pH 4.0.

Substrate	Before incubation		After incubation		Probable transformation
	λ min. (mμ)	λ max. (mμ)	λ min. (mμ)	λ max. (mμ)	
A	228	261	228	261	(A→A)
AR	228	259	223	250	(AR→Hx)
A3P	227	258	222	250	(A3P→Hx)
Hx	222	250	222	250	(Hx→Hx)
HxR	222	249	222	250	(HxR→Hx)
G	225	247	225	247	(G→G)
GR	223	252	225	249	(GR→G)
G3P	223	252	223	249	(G3P→G)
X	240	265	240	265	(X→X)
U	227	259	227	259	(U→U)
UR	230	261	230	261	(UR→UR)
U3P	230	261	230	261	(U3P→UR)
C	239	273	239	273	(C→C)
CR	243	275	243	275	(CR→CR)
C3P	242	275	242	275	(C3P→CR)
T	233	264	233	264	(T→T)

Composition of reaction mixture: 0.02 ml of enzyme preparation and 5 ml of dilute solution of substrate in N/20 acetate buffer, pH 4.0. Incubation time: 18 hr., temp.: 45°C.

the changes in ultraviolet absorption spectra of these reaction mixtures during incubation. If the absorption spectrum does not change during incubation, it is apparent that the substrate is not converted to any compound with a different spectrum. Thus under the present conditions adenine, hypoxanthine, guanine, xanthine, uracil, uridine, cytosine, cytidine and thymine were not subjected to any enzymic action. Although the ultraviolet absorption spectra of 3′-uridylic and 3′-cytidylic acids did not change in form during incubation, it is clear from the results described in "2. i, ii", that 3′-uridylic and cytidylic acids are converted to their nucleosides by the *Aspergillus oryzae* enzyme. The slight differences of λ max between Tables 5 and 6 may be most probably based on the difference of pH. This series of experiments shows clearly that the ribonucleolytic enzyme system from *Aspergillus oryzae* var. No. 13 contains mononucleotide phosphatase, purine nucleosidase, and adenyl deaminase as well as ribonucleodepolymerase.

As described in "(b) 1", the degradation products resulting from the action of the ribonucleolytic enzyme system on yeast ribonucleic acid were identified to be adenosine, guanosine, cytidine and uridine. Other products were not recognized. However, it is suggested from the results in "(b)

2 and (c)" that under more appropriate conditions hypoxanthine, guanine, (guanosine,) cytidine, and uridine may be recognized as the end products resulting from the action of the *Aspergillus oryzae* ribonucleolytic enzyme system on yeast ribonucleic acid. This point is being investigated.

In the next section the mode of splitting of the nucleosidic linkage by *Aspergillus oryzae* enzymes is discussed in some detail.

(3) *Proof of the existence of purine nucleoside hydrolase in Aspergillus oryzae.*

The phosphorolytic cleavage of the glycosidic linkage in purine and pyrimidine nucleosides was first demonstrated with inosine and guanosine by Kalckar[8],[20] in rat liver preparations. Carter[21], however, furnished the first evidence for the conclusion that not all nucleosidases are phosphorylases, but that hydrolytic enzymes for the cleavage of the bonds between purine or pyrimidine bases and ribose do exist.

The existence of purine nucleosidase in *Aspergillus oryzae* was demonstrated in the foregoing experiments. In the present experiment evidence is provided which established the action of the *Aspergillus* enzyme as a hydrolytic cleavage at the ribosidic linkage in inosine or guanosine.

The enzyme preparations used in this section were obtained as follows: Culture filtrates of *Aspergillus oryzae* var. No. 13 were concentrated *in vacuo*. The concentrated filtrates were stored in a deep freeze for a few days. After removal of any precipitate, the resulting supernatant solution was dialyzed against M/50 sodium acetate solution, and then was subjected to salting out with ammonium sulfate. Preparation A (optical density*:8.13), the fraction precipitated between 0.4 and 0.6 saturated ammonium sulfate, was rich in adenyl deaminase, but poor in the other ribonucleolytic enzymes. The phosphatase in this preparation was almost completely inhibited by M/100 sodium fluoride. Preparation B (optical density*: 4.82), the fraction precipitated between 0.6 and 0.8 saturated ammonium sulfate was rich in depolymerase, phosphatase and nucleosidase. Preparation C (optical density*: 0.73), the fraction precipitated between 0.8 and 1.0 saturated ammonium sulfate, contained a relatively strong activity of nucleosidase, but only slight activities of the other enzymes. Preparation D (optical density*:13.52), the fraction precipitated between 0.5 and 1.0 saturated ammonium sulfate, was obtained from another culture filtrate. Enzymic activities and purities of this preparation were lower than those of preparations A, B, and C, but the various enzymes were rather evenly distributed. These enzyme preparations do not contain any significant amount of inorganic phosphate and pyrophosphate. Incubation without enzyme did not cause any degradation of the various nucleosides.

(a) *Influence of inorganic phosphate on the enzymic splitting of inosine.*

As shown in Table 7, the rate of enzymic splitting of inosine by *As-*

* Optical densities were read at 275 mμ and pH 4.4.

pergillus oryzae var. No. 13 in phosphate buffer was essentially equal to that in acetate buffer. Sodium fluoride did not significantly influence the splitting of inosine in phosphate buffer or in acetate buffer. The hydrolytic reaction proceeded irreversibly. The sugar, formed as one of the products, was characterized as ribose, but not as ribose-1-phosphate by paper chromatography. Moreover, the results in Table 8 show that the amount of start-

Table 7. Influence of phosphate and sodium fluoride on enzymic splitting of inosine.

	Buffer	Phosphate (pH 5.15)		Acetate (pH 4.80)	
	NaF (M/100)	−	+	−	+
Formation of Hx (%)*	0 min.	0	0	0	0
	30 min.	43.1	50.0	46.7	46.7
	60 min.	83.8	88.6	84.4	87.4
	120 min.	100.0	100.0	100.0	100.0
	R_F values** of reducing sugar formed	0.47	0.47	0.47	0.47

Composition of reaction mixture: 0.04 ml of substrate soln. (HxR, 1.50 μ moles), 0.02 ml of enzyme preparation C, 0.05 ml of N/10 phosphate or acetate buffer, and 0.02 ml of sodium fluoride or water. Total volume 0.13 ml

Incubation temp.: 45°C.

* (Hx formed) × 100/HxR added.

** The R_F values in solvent (3) of standard substances were as follows: ribose, 0.47; R1P, 0.29.

Table 8. Enzymic splitting of inosine in the presence of phosphate.

Time (min.)		
Compd. in reaction mixture	0	60
	μ moles	μ moles
HxR	19.2	4.1
Inorg. P	16.6	17.0
Reducing sugar*	0	14.0
Hx	0	15.1

Composition of reaction mixture: 0.5 ml of substrate soln., 0.2 ml of enzyme preparation C, 0.5 ml of N/10 phosphate buffer, pH 5.15, 0.2 ml of M/10 sodium fluoride, and 0.6 ml of M/10 acetate buffer, pH 4.80.

Incubation temp.: 45°C. Total volume 2.0 ml.

* The R_F value (0.47) in solvent (3) was similar to that of ribose.

ing inorganic phosphate was not changed during incubation, and equimolecular amounts of ribose and hypoxanthine were formed from inosine. Results essentially similar to those in Tables 7 and 8 have been obtained with preparations A, B, and D.

Thus it is concluded that the splitting of inosine in *Aspergillus oryzae* var. No. 13 is carried out by a nonphosphorolytic hydrolase as follows:

$$\text{Inosine} + H_2O \rightarrow \text{Hypoxanthine} + \text{Ribose} \qquad (6)$$

Experiments with guanosine as a substrate gave similar results.

$$\text{Guanosine} + H_2O \rightarrow \text{Guanine} + \text{Ribose} \qquad (7)$$

(b) *Substrate specificity of nucleoside hydrolase*

The rate of splitting of guanosine by nucleoside hydrolase from *Aspergillus oryzae* var. No. 13 was about 30% of that of inosine. In the experiments so far carried out complete splitting of guanosine has not been observed.

The ribosidic linkages of adenosine, cytidine, and uridine were not split by the enzyme. Activity towards uridine and cytidine was also not observed when *Aspergillus oryzae* var. No. 13 was grown on a medium containing 300 μg/ml of uridine or cytidine in addition to the basic components. These results will be described in more detail elsewhere.

In view of the experiments described above, at any rate, it may be indicated that *Aspergillus oryzae* var. No. 13 has a purine nucleoside hydrolase activity towards inosine and guanosine, but is inactive towards adenosine, cytidine, and uridine.

(Although the culture filtrates of *Aspergillus oryzae A*, and *Aspergillus niger a* were observed also to be capable of hydrolyzing inosine to ribose and hypoxanthine, those of *Rhizopus nigricans* NRRL No. 45, *Mucor spinescens* Mu-3, and *Monascus purpureus* 3–1 were not. The results are shown in Table 12.)

(4) *Proof of the existence of 5′-inosinate-N-ribosidase in Aspergillus oryzae.*

According to the prevailing theory, the phosphoric acid group in the nucleotides has to be removed before the ribosidic linkage can be resolved by enzymes. Levene and Dmochowski[22], using nucleosidase prepared from pig small intestine, came to the conclusion that the ribosidic linkage of guanylic or adenylic acid was not broken until the phosphate was removed. However, Schmidt[23] found that a watery extract of rabbit liver attacked guanylic acid, producing ammonia, purine, and ribose phosphate. Furthermore Ishikawa and Komita[24] discovered that dog pancreas was capable of disrupting the glucosidic linkages of guanylic and xanthylic acids without the previous cleavage of phosphate. Komita[25,26] has separated this enzyme from purine nucleosidase, and named it nucleotide-N-ribosidase. Lately Schlenk[27] indicated another possible interpretation of these experiments in the

light of more recent observations. According to his interpretation, guanylic acid may be split first by phosphatase. The resulting guanosine is cleaved by phosphorolysis, and the resulting ribose-1-phosphate is rearranged by mutase action to ribose-5-phosphate and to hexose-6-monophosphate and other esters. Other experiments of MacFadyen[14] suggested that the nucleotides were disintegrated by *Bacillus subtilis* mainly to phosphoric acid esters of ribose and nitrogenous bases, but the details of this reaction were not described. At any rate proof of the existence of 3′-nucleotide-N-ribosidase is lacking, at present. Regarding 5′-nucleotide-N-ribosidase, there is not any clear evidence for its existence.

In the present experiments it was observed that 5′inosinic acid is split by the enzyme preparation of *Aspergillus oryzae* var. No. 13 to yield hypoxanthine and ribose-5-phosphate. Morever this reaction is not carried out by the successive action of mononucleotide phosphatase, nucleoside phosphorylase, and phosphoribomutase, but is carried out by the action of an enzyme hydrolyzing the ribosidic linkage of 5′-inosinic acid directly.

The enzyme preparations used in the foregoing experiment "(3)" were employed also in the present experiments. Incubation was carried out at 45°C and pH 4.7–5.2. Under these conditions, nucleotides and inosine were not degraded without enzyme for 4 hours at least. In the case of 5′-inosinic acid, its stability was ascertained for 10 hours.

(a) *Enzymic degradation of 3′- and 5′-inosinic acids.*

3′-Inosinic acid was degraded by the enzymes of *Aspergillus oryzae* to inorganic phosphate, reducing sugar, inosine, and hypoxanthine. These products were observed also in the case of the degradation of 5′-inosinic acid. Of these product, inosine was not recognized when the activity of nucleoside hydrolase was much stronger than that of phosphatase. Because inosine was not formed from hypoxanthine as described in "(3)", it might be formed from 3′- or 5′-inosinic acid directly by phosphatase. Therefore the existence of the following pathways is apparent:

$$\text{3′-Inosinic acid} + H_2O \rightarrow \text{Inosine} + \text{Inorganic phosphate} \quad (8)$$

$$\text{Inosine} + H_2O \rightarrow \text{Hypoxanthine} + \text{Ribose} \quad (6)$$

$$\text{5′-Inosinic acid} + H_2O \rightarrow \text{Inosine} + \text{Inorganic phosphate} \quad (9)$$

$$\text{Inosine} + H_2O \rightarrow \text{Hypoxanthine} + \text{Ribose} \quad (6)$$

In the reaction with 3′-inosinic acid, the amount of reducing sugar formed was always less than that of inorganic phosphate formed, and the R_F value of the reducing sugar was similar to that of ribose. The sum of inosine and hypoxanthine essentially equaled inorganic phosphate. Moreover, sodium fluoride inhibited not only the formation of inorganic phosphate and inosine but also that of hypoxanthine and reducing sugar. A representative experiment is shown in the left-hand columns in Table 9. These results indicate

Table 9. Enzymic degradation of inosinic acid with and without sodium fluoride.

	Substrate	I3P (5.25 μ moles)		I5P (6.90 μ moles)	
	NaF (M/100)	−	+	−	+
Products (μ moles)	Inorg. P	1.50	0	2.00	0
	Reducing sugar	0.97	0	2.70	2.25
	HxR	0.65	0	1.45	0
	Hx	0.94	0	2.62	2.32
	R_F* of reducing sugar formed	0.47	—	0.19 0.47	0.19 [0.47 (trace)]

Composition of reaction mixture: 0.25 ml of substrate soln., 0.20 ml of enzyme preparation A, 0.10 ml of M/10 sodium fluoride or water, and 0.45ml of M/10 acetate buffer, pH 4.75. Total volume 1.0 ml. Incubation temp.: 45°C, time: 120 min.

* The R_F vales in solvent (3) of standard substances were as follows: ribose, 0.47; R3P, 0.29; R5P, 0.19.

that 3′-inosinic acid was degraded first to inosine and inorganic phosphate by phosphatase sensitive to sodium fluoride (Eq. (8)), and next the resulting inosine was split to hypoxanthine and ribose by nucleoside hydrolase insensitive to sodium fluoride (Eq. (6)). The other degradation pathways, therefore, are very improbable.

On the other hand, the reaction with 5′-inosinic acid was different from that with 3′-inosinic acid. In this case the ratio of reducing sugar to inorganic phosphate in the reaction mixture was always more than 1. The spot of ribose phosphate, as well as that of ribose, was detected on the paper. Furthermore, although the formation of inosine and inorganic phosphate was inhibited strongly by sodium fluoride, that of hypoxanthine and reducing sugar was only slightly influenced by this inhibitor. Most of the reducing sugar formed from 5′-inosinic acid in the presence of fluoride was not ribose, but ribose phosphate. An example of these results is shown in the right-hand columns in Table 9. Similar results were obtained with preparations B, C, and D. Accordingly it is suggested that the degradation of 5′-inosinic acid is carried out not only by the mechanism as shown in Eq. (9) and (6), but also by another unknown mechanism.

(b) *Separation and properties of ribose phosphate formed from 5′-inosinic acid.*

To clarify this unknown mechanism of degradation of 5′-inosinic acid, the properties of the ribose phosphate formed from 5′-inosinic acid in the presence of fluoride were investigated. A solution (8.0 ml) containing 41.5 μ moles of 5′-inosinic acid (1.5 ml), 2 ml of enzyme preparation D, 3.7 ml of M/10 acetate buffer, pH 4.75, and 0.8 ml of M/10 sodium fluoride was incubated at 45°C. When the formation of hypoxanthine from 5′-inosinic acid

arrived at 100%†, cold perchloric acid (final concentration : 2%) was added to 5 ml of the reaction mixture. After the resulting solution was cooled and adjusted to pH 4.0, hypoxanthine was removed as a precipitate of silver salt by addition of silver sulfate to the solution. The precipitate was centrifuged and washed. The solution and wash water were combined and treated with hydrogen sulfide. After removal of the excess of hydrogen sulfide, the mixture was filtered and the precipitate was washed with hot water. The solution was adjusted to pH 6–7 with saturated barium hydroxide solution. After concentration *in vacuo*, more barium hydroxide was used to adjust the pH to 8.5. The precipitate was centrifuged down and washed repeatedly with small amounts of water. The combined solutions were precipitated slowly with three volumes of ethanol. After standing, the barium salt of the ester was centrifuged, washed with ethanol, and dried in a desiccator. It was dissolved in a small amount of water, and the treatment with ethanol was repeated.

The ratio of the ribose component to the phosphate component was found to be 1.01. The hydrolysis rate in hydrochloric acid, the R_F value, the electrophoretic mobility, and the rate of orcinol reaction of this ribose phosphate preparation were essentially similar to those of ribose-5-phosphate, and were distinguished from those of ribose-1-phosphate or ribose-3-phosphate. These results are shown in table 10 and figure 13. (The electrophoretic mobility of ribose-1-phosphate was essentially similar to that of ribose. Although it is difficult to determine whether this value is that of ribose-1-phosphate itself or that of ribose formed during the electrophoresis procedure, at any rate the mobility of ribose-5-phosphate was clearly distinguished. The rates of orcinol reaction with both ribose and ribose-5-phosphate

Table 10. Properties of the ribose phosphate formed from 5'-inosinic acid.

		% Hydrolysis in 1N HCl at 100°C		R_F in solvent (3)	Distance*** migrated from origin by electrophoresis (cm)
		7 min.	180 min.		
Ribose phosphate formed		1.22 (?)	60.9	0.18	+13.2
Standard	R5P	2.74	59.1 (58.7)*	0.18	+13.0
	R1P	(100)*	(100)*	0.28	+ 3.8 (?)
	R3P	**	**	0.29	+13.8
	Ribose			0.46	+ 3.5

* The values in brackets are according to Le Page[28]. The value for phosphocreatine was used in place of that of R1P which is absent from the literature, for R1P is more labile than phosphocreatine[23].

** R3P hydrolyzes 5 to 9 times faster than R5P[29].

*** Caffeine: +3.5 cm.

† The ratio of hypoxanthine formed was determined by paper electrophoresis for short time.

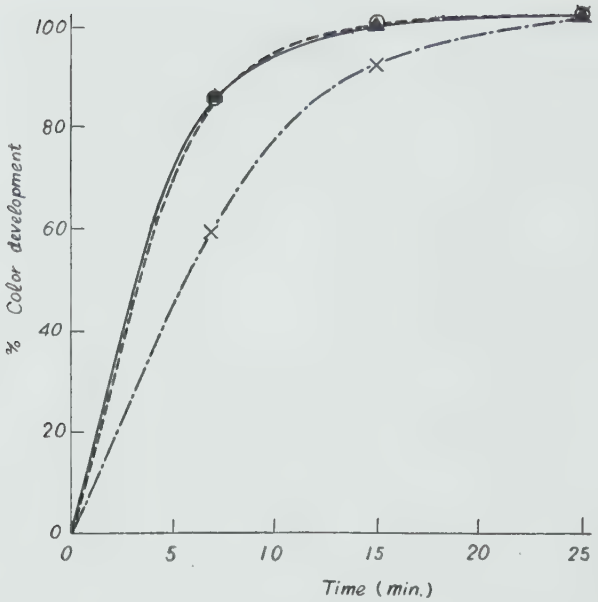

Fig. 13. Rate of color development in the pentose-orcinol reaction,

—▲— ribose phosphate formed
– – –○– – – R5P
·–·–×–·–· ribose

The technique employed was essentially the same as that described by Albaum and Umbreit[30].

(The curves of R1P and R3P are similar to that of ribose.)

were relatively larger than the results obtained by Albaum and Umbreit[30]. This difference may be based on the difference of reagent used). From these results the ribose phosphate formed with hypoxanthine from 5′-inosinic acid is characterized as ribose-5-phosphate.

This observation suggests that the following reaction, insensitive to sodium fluoride, does exist:

$$\text{5′-Inosinic acid} \rightarrow \text{Hypoxanthine} + \text{Ribose-5-phosphate} \qquad (10)$$

There appear to be two possible mechanisms to account for this equation:

i. Direct splitting of ribosidic linkage

$$\text{5′-Inosinic acid (hypoxanthine}\text{—}_{1}\text{ribose}_{5}\text{—phosphate)} + H_2O \longrightarrow \text{Hypoxanthine} + \text{Ribose-5-phosphate} \qquad (11)$$

ii. Action in succession by phosphatase, phosphorylase and phosphoribomutase

$$\text{5′-Inosinic acid} + H_2O \rightarrow \text{Inosine} + \text{Inorganic phosphate} \qquad (9)$$

$$\text{Inosine} + \text{Inorganic phosphate} \rightleftarrows \text{Hypoxanthine} + \text{Ribose-1-phosphate} \quad (12)$$

$$\text{Ribose-1-phosphate} \rightleftarrows \text{Ribose-5-phosphate} \quad (13)$$

Sum: 5′-Inosinic acid$+H_2O \rightarrow$Hypoxanthine$+$Ribose-5-phosphate.

Although mechanism (ii) is more probable than mechanism (i) in animal tissue, there is little possibility of its existence in *Aspergillus oryzae* because of the following reasons: Formation of hypoxanthine and ribose-5-phosphate is little influenced by inhibition of phosphatase action (Eq. (9)), and nucleoside phosphorylase action (Eq. (12)) is not recognized under the present conditions.

In order to make certain, however, phosphoribomutase action (Eq. (13)) in *Aspergillus oryzae* was investigated.

(c) *Proof of the absence of phosphoribomutase action.*

0.1 ml of a crude preparation of ribose-1-phosphate (prepared from 2.5 mg of inosine) was adjusted to pH 5.0 and incubated with 0.02 ml of M/10 sodium fluoride and 0.06 ml of enzyme preparation D at 45°C for 2 hours. During incubation the R_F value (0.29) and electrophoretic mobility (+4.0 cm) of ribose-1-phosphate did not change. (At the same time formation of ribose [$R_F = 0.45$] was also observed. This ribose is probably formed from inosine, present in the substrate solution, by the nucleoside hydrolase of *Aspergillus oryzae*.) In another experiment the reaction mixture was treated with an equal volume of 2N hydrochloric acid at 100°C for 7 minutes, dried over potassium hydroxide, and then subjected to paper chromatography. A spot of ribose phosphate was not recognized, but one of ribose was recognized. This result was obtained both with and without the enzyme reaction. These observations indicate that *Aspergillus oryzae* enzymes do not catalyze the transformation of acid-labile ribose-1-phosphate into acid-stable ribose-5-phosphate.

Under conditions such as described above, the action of enzyme preparation D on ribose-5-phosphate was studied. During incubation with enzyme preparation D the R_F value (0.17) and electrophoretic mobility (+13.5 cm) of ribose-5-phosphate did not change. After the treatment with hydrochloric acid, the reaction mixture was observed to contain not ribose, but to contain only ribose phosphate. This result was obtained both with and without the enzyme reaction. These observations indicate that *Aspergillus oryzae* enzymes do not catalyze the transformation of acid-stable ribose-5-phosphate into acid-labile ribose-1-phosphate.

Thus phosphoribomutase action (Eq. (13)) was not recognized in *Aspergillus oryzae* var. No. 13 under the present experimental conditions.

Therefore the 5′-inosinic acid degradation mechanism (ii), action in succession by phosphatase, phosphorylase and phosphoribomutase, was not recognized in *Aspergillus oryzae* var. No. 13, at least in the presence of sodium fluoride. This apparently indicates that mechanism (i), the direct splitting reaction of the ribosidic linkage of 5′-inosinic acid, exists in *Aspergillus*

oryzae var. No. 13.

(d) *Enzymic action on the ribosidic linkage in various mononucleotides.*

In the presence of sodium fluoride various nucleotides were incubated with enzyme preparation C. The results are shown in Table 11. The for-

Table 11. Enzymic action upon ribosidic linkage in various mononucleotides.

Substrate (μ moles)		Degradation Products (μ moles)		
		Base	Reducing sugar	Inorg. P.
A3P	8.00	0	0	0.38
A5P	6.83	0	0	0.45
I 5P	4.95	4.12	4.2	0.28
G3P	9.33	+	0.2	0.56
C3P	8.85	0	0	0.45
U3P	10.52	0	0	0.33

Composition of reaction mixture: 0.25 ml of substrate, 0.15 ml of enzyme preparation C, 0.10 ml of M/10 sodium fluoride, and 0.50 ml of M/10 acetate buffer, pH 4.8. Total volume 1.00 ml. Incubation time: 30 min. temp.: 45°C.

mation of reducing sugar and base was observed only from 3′-guanylic acid and 5′-inosinic acid. From 3′- or 5′-adenylic, 3′-cytidylic, and 3′-uridylic acids, small amounts of the corresponding nucleosides and inorganic phosphate were formed by the action of a trace of phosphatase. As the enzyme preparation used was free from adenyl deaminase, deamination of 3′- or 5′-adenylic acid, and adenosine was not observed. The formation of guanine and reducing sugar from 3′-guanylic acid strongly depended on the liberation of inorganic phosphate, and furthermore the ratio of reducing sugar to inorganic phosphate was always less than 1. Thus guanine is probably liberated via guanosine. Direct liberation of xanthine from 3′-xanthylic acid by *Aspergillus oryzae* was not recognized qualitatively.

In conclusion direct hydrolysis of the ribosidic linkage in the mononucleotides investigated is carried out by *Aspergillus oryzae* in 5′-inosinic acid only. Accordingly it seems that "5′-inosinate-N-ribosidase" might be an appropriate name for this enzyme.

(e) *Influence of inorganic orthophosphate and pyrophosphate on the enzymic splitting of the ribosidic linkage of 5′-inosinic acid.*

The series of experiments described above was carried out without the presence of any appreciable amounts of inorganic ortho- or pyrophosphate. Moreover, direct liberation of hypoxanthine from 5′-inosinic acid proceeded irreversibly. Therefore this reaction is catalyzed probably by a nonphosphorolytic hydrolase. This concept is supported by the following preliminary

experiment.

The partially purified enzyme preparation was incubated with 5′-inosinic acid (4.80 μ moles) in the presence of inorganic orthophosphate (10.0 μ moles) or pyrophosphate (5.0 μ moles) at 45°C and pH 5.0. (Total volume 1.0 ml). The amounts of hypoxanthine formed after 1 hour were as follows: without phosphates, 0.90 μ moles; with orthophosphate, 0.99 μ moles; with pyrophosphate, 0.69 μ moles. The enzyme preparation used in this preliminary experiment had only slight activity, and further studies are needed. However, it is conceivable that 5′-inosinate-N-ribosidase is not activated to any appreciable extent by inorganic orthophosphate or pyrophosphate. There is no evidence so far that 5′-inosinate-N-ribosidase is either a phosphorylase or a pyrophosphorylase.

(f) *Action of several molds on the ribosidic linkage of 5′-inosinic acid or inosine.*

Several kinds of molds were cultured under the conditions employed in foregoing experiments. Each culture filtrate was incubated with 5′-inosinic acid or inosine in the presence of sodium fluoride. Two strains* which belong to the *Aspergillus* genus, as well as *Aspergillus oryzae* var. No. 13, were observed to be capable of splitting the ribosidic linkage of 5′-inosinic acid or inosine. By contrast *Rhizopus nigricans**, *Mucor spinescens**, and *Monascus purpureus** were observed to be incapable of splitting these compounds at least under the present conditions. The results are summarized in Table 12.

It may be suggested that the new enzyme "5′-inosinate-N-ribosidase," as well as purine nucleoside hydrolase, is distributed among the strains which

Table 12. Enzymic formation of hypoxanthine from 5′-inosinic acid or inosine by several molds (μ moles).

Strain \ Substrate	I5P	HxR
Asp. oryaze var. No. 13	0.31	0.64
Asp. oryzae A	0.29	0.11
Asp. niger a	0.24	0.19
Rhizopus nigricans NRRL No. 45	0	0
Mucor spinescens Mu-3	0	0
Monascus purpureus 3–1	0	0

Composition of reaction mixture: 0.04 ml of substrate soln. (1.46 μ moles), 0.03 ml of enzyme soln. (culture filtrates), 0.01 ml of M/10 sodium fluoride, and 0.02 ml of M/10 acetate buffer, pH 5.0. Total volume 0.10 ml. Incubation time: 60 min., temp.: 45°C.

* I wish to express my thanks to Assoc. Prof. H. Iizuka, the Institute of Applied Microbiology, University of Tokyo, for kindly supplying these strains for this study.

belong to the *Aspergillus* genus. The investigation of this possibility will be the subject of future research.

DISCUSSION

From the results of the present study, the principal pathways in the enzymic degradation of yeast ribonucleic acid and its related compounds by a strain of Koji-molds, *Aspergillus oryzae* var. No. 13, are established as shown in Figure 14. The properties of the ribonucleolytic enzymes responsible for the reactions in these pathways will now be discussed mainly from the viewpoint of comparative biochemistry.

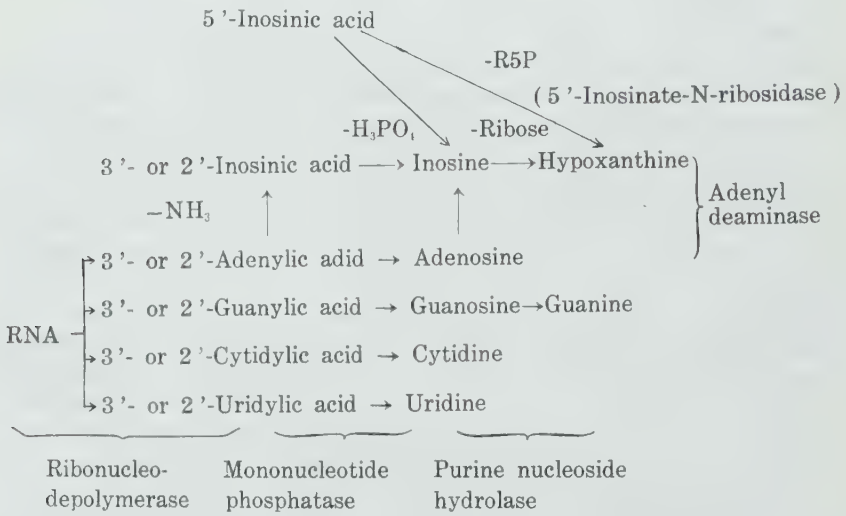

Fig. 14. Principal pathways in the enzymic degradation of RNA by *Aspergillus oryzae* var. No. 13 (pH 4.0~5.2).

(1) *Ribonucleodepolymerase*

While the mononucleotide products obtained after exhaustive digestion of ribonucleic acid with pancreas ribonuclease I are 3′-cytidylic and 3′-uridylic acids, those with the ribonucleodepolymerase of *Aspergillus oryzae* var. No. 13 are 3′-(or 2′-) adenylic, 3′-(or 2′-) guanylic, 3′-(or 2′-) cytidylic, and 3′-(or 2′-) uridylic acids. This finding supports the view that the ribonucleodepolymerase of *Aspergillus oryzae* catalyzes the cleavage of the internucleotide bonds between the 3′-purine or pyrimidine nucleoside phosphoryl groups and the 5′-hydroxy groups of the adjacent purine or pyrimidine nucleotide groups. Thus the specificity towards the internucleotide bonds of the enzyme is wider than that of pancreas ribonuclease I, and is rather similar to that of degradation by alkali. In regard to the nonspecific for-

mation of four mononucleotides from ribonucleic acid, this enzyme resembles snake venom phosphodiesterase. However, in contrast to the action of *Aspergillus* ribonucleodepolymerase, snake venom diesterase causes specifically the cleavage of the 3′-ester linkages between the phosphoryl groups and the corresponding nucleoside residues.

Aspergillus ribonucleodepolymerase is rather heat-stable as well as pancreas ribonuclease I. While the former is especially stable in the region of pH 6.0, the latter is stable in the region of pH 3.0.

Aspergillus ribonucleodepolymerase differs from pancreas ribonuclease I also by the different pH range of its optimal activity. While the pH optimum of the former is at about 4.0, that of the latter is at 7.7. Moreover, monoiodoacetate, ninhydrin, and potassium cyanide were without appreciable influence on this enzyme. The electrophoretic properties of this enzyme protein are being investigated[31].

Spleen ribonuclease[32,33] and mouse ascites tumor ribonuclease[34] resemble this enzyme in regard to the specificity towards the internucleotide bonds, and to the pH optimum. It is especially interesting that the tumor enzyme is also comparatively thermostable. Although the *Aspergillus* enzyme seems to be more thermostable than the tumor enzyme, these two enzymes are remarkably related to one another. On the other hand the spleen enzyme is thermolabile.

Whether the ribonucleodepolymerase activity of *Aspergillus oryzae* is due to one or more enzyme proteins remains an open question. However, it is probable that *Aspergillus oryzae* var. No. 13 contains a new ribonucleodepolymerase other than the enzymes obtained so far from other sources.

It seems that the concept described above is supported by the short communications on crystallization of Takadiastase ribonuclease from Saruno[35].

(2) *Mononucleotide phosphatase*

It has been demonstrated that the enzymic liberation of inorganic phosphate by *Aspergillus oryzae* is carried out after ribonucleic acid is degraded into four 3′- or 2′-mononucleotides by ribonucleodepolymerase, and that four nucleosides are formed with liberation of inorganic phosphate. Thus the ribonucleophosphatase in *Aspergillus oryzae* var. No. 13 can be regarded as a mononucleotide phosphatase. This enzyme is probably related to the nonspecific acidic phosphomonoesterase from plants, because its activity is not influenced by magnesium ion and arsenate, but inhibited by sodium fluoride, and its optimum pH is near 4.0; furthermore, its activity towards mononucleotides is in parallel with that towards β-glycerophosphate. The details of such experiments will be published elsewhere. The activity towards 5′-mononucleotides will be the subject of future research.

The simple method devised for simultaneous detection of the enzymes in the ribonucleolytic enzyme system splitting phosphate bonds may also be applied to the detection of only mononucleotide phosphatase, by giving an appropriate substrate in place of ribonucleic acid.

(3) *Ribonucleodeaminase*

The enzymic deamination of 3′-adenylic acid and adenosine by *Aspergillus oryzae* was established. Kaplan *et al*[36] reported that the deaminase from Takadiastase also deaminates 5′- and 3′-adenylic acids, diphosphopyridine nucleotide, adenosine diphosphate ribose, and adenosine diphosphate, as well as adenosine. Thus it is probable that in contrast to the specific deaminase from animals, nonspecific adenyl deaminase is distributed among the strains which belong to the *Aspergillus* genus.

Enzymic deamination of adenine, guanylic acid, guanosine, guanine, cytidylic acid, cytidine, and cytosine were not recognized under the present conditions. The transformation of adenosine to hypoxanthine by *Aspergillus oryzae* via inosine but not adenine may be similar to that occurring in mammalian tissues.

(4) *Ribonucleosidase*

Aspergillus oryzae contains a nonphosphorolytic purine nucleoside hydrolase acting on inosine and guanosine but not adenosine. The nucleoside hydrolases found so far in *Lactobacillus pentosus*[37], yeast[38], and fish muscle[10] show rather wide specificity, and are capable of splitting adenosine also. On the other hand, most of the nucleoside phosphorylases described so far are incapable of splitting adenosine. Thus purine nucleoside hydrolase from *Aspergillus oryzae* is rather similar to nucleoside phosphorylase in so far as its specificity is concerned.

Pyrimidine nucleosidase activity was not recognized in *Aspergillus oryzae* even when cytidine or uridine was added to the culture medium. In contrast to the case of purines, pyrimidine nucleosides are generally known to be more readily assimilated than the corresponding bases. Therefore the lack of pyrimidine nucleosidase activity in *Aspergillus oryzae* does not seem so unnatural.

(5) *5′-Inosinate-N-ribosidase*

That purines might incorporated directly into nucleotides, rather than first forming nucleosides that are then phosphorylated, has been suggested by Saffran[39], Kornberg[40], and Buchanan[41] et al. Saffran and Scarano[39] proposed the following equation:

$$\text{Ribose-1, 5-diphosphate} + \text{Adenine} \rightarrow 5'\text{-Adenylic acid} + \text{Inorganic phosphate} \qquad (14)$$

Kornberg[40] and Buchanan[41] proposed the following equation:

$$\text{Base} + \text{5-Phosphoribosylpyrophosphate} \rightleftarrows 5'\text{-Nucleotide} + \text{Inorganic pyrophosphate} \qquad (15)$$

(Base: adenine, hypoxanthine, guanine, orotic acid.)

The reverse reaction of Eq. (14) or (15) offers direct liberation of base from the 5′-nucleotide, The enzyme "5′-inosinate-N-ribosidase", demonstrated in

Aspergillus oryzae in the present study, specifically catalyzes an irreversible hydrolysis of 5′-inosinic acid. Thus it is distinguished from nucleotide-1′-phosphorylase (Eq. (14)) or nucleotide-1′-pyrophosphorylase (Eq. (15)). Therefore the enzymic cleavage of the ribosidic linkage in 5′-inosinic acid is accomplished by a dual mechanism: (pyro) phosphorolysis and hydrolysis. This fact is interesting because a similar dual mechanism has been established for the cleavage of nucleosides. This enzyme also differs clearly from 3′-nucleotide-N-ribosidase described by Komita[24-26] in regard to specificity. As for the splitting mechanism, this enzyme resembles diphosphopyridine nucleotidase[42].

Moreover, from the fact that 5′-inosinate-N-ribosidase was found in *Aspergillus oryzae* containing purine nucleoside hydrolase, the distribution of such an enzyme (5′-nucleotide-N-ribosidase) among various organisms containing nucleoside hydrolase is probable. (The properties of 5′-inosinate-N-ribosidase resemble those of purine nucleoside hydrolase. Separation of these enzymes has not been accomplished. Thus at present it is not certain whether liberation of hypoxanthine from inosine and from 5′-inosinic acid is caused by two independent enzyme proteins or by one enzyme protein. Further experiments on this point are being carried out).

Although the biochemical significance of 5′-inosinate-N-ribosidase action is not clear, it is interesting that this enzyme attacks specifically 5′-inosinic acid regarded as a key intermediate. With this enzyme, the reactions responsible for formation of hypoxanthine from 5′-inosinic acid, 5′-phosphoribosyl-5-amino-4-imidazolecarboxamide, 5′-adenylic acid and its derivatives, may be shortened by one step, and may offer a new source of ribose-5-phosphate.

The conclusions reached in this study will be verified with more highly purified preparations of the respective enzymes.

SUMMARY

The principal pathways in the enzymic degradation of yeast ribonucleic acid by *Aspergillus oryzae* var. No. 13 have been established as shown in Figure 14, mainly by means of characterization of enzymic degradation products. Paper electrophoresis, paper chromatography, and ultraviolet spectrophotometry were employed for characterization.

Ribonucleodepolymerase degrades yeast ribonucleic acid into 3′ (or 2′)-adenylic, 3′ (or 2′)-guanylic, 3′ (or 2′)-cytidylic, and 3′ (or 2′)-uridylic acids without liberation of inorganic phosphate. It does not degrade sperm deoxyribonucleic acid. The activity is still preserved after dialysis and contact with weak-base anion exchange resin (Amberlite IR-4B) or Japanese acid clay. This enzyme is remarkably thermostable, especially in the range of pH from 5.5 to 6.5. In 0.05N sodium acetate solution 94% of the activity is retained after heating for 10 minutes at 100°C. The optimum condi-

tions for the activity are at about 60°C and pH 4.0. This enzyme is not influenced by ninhydrin, monoiodoacetate, potassium cyanide, as well as sodium fluoride.

Mononucleotide phosphatase is thermolabile, and readily inactivated by physical or chemical treatments. Evidence suggests that this enzyme has properties similar to those of the general acidic phosphomonoesterase of plant origin. A brief procedure has been devised for the simultaneous detection of ribonucleodepolymerase and mononucleotide phosphatase on an agar plate.

The splitting of inosine and guanosine is carried out by nonphosphorolytic hydrolase. Adenosine, cytidine, and uridine are not split by the *Aspergillus oryzae* enzyme.

The direct liberation of hypoxanthine from 5′-inosinic acid by hydrolysis was first recognized. The enzyme responsible for this reaction is a nonphosphorolytic hydrolase and attacks 5′-inosinic acid specifically. Thus it seems that "5′-inosinate-N-ribosidase" might be an appropriate name for this enzyme.

ACKNOWLEDGEMENTS

I wish to express my hearty thanks to Prof. K. Sakaguchi, Director of the Institute of Applied Microbiology, without whose constant guidance and inspiration this project would have been impossible. I am also indebted to Assoc. Prof. K. Arima, University of Tokyo, for his valuable advice throughout, to Dr. M. Onuki and Mr. S. Kibi for their constant encouragement, and to Miss K. Suzuki for her assistance in carrying out the experiments.

This communication includes the results of the following reports which have been published in Japanese, plus several unpublished results.

"Studies on the decomposition of nucleic acid by microörganisms."

Part 1, *J. Agr. Chem. Soc.* Japan., **28**, 282 (1954)., Part 2, *ibid.*, **29**, 52 (1955)., Part 3, *ibid.*, **29**, 797 (1955)., Part 4, *ibid.*, **29**, 801 (1955)., and Part 5, *ibid.*, **30**, 583 (1956).

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Printed at
Kokusai Bunken Insatsusha
Chiyoda-ku, Tokyo

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